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SCIENTIFIC EDITION

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This issue in two Parts

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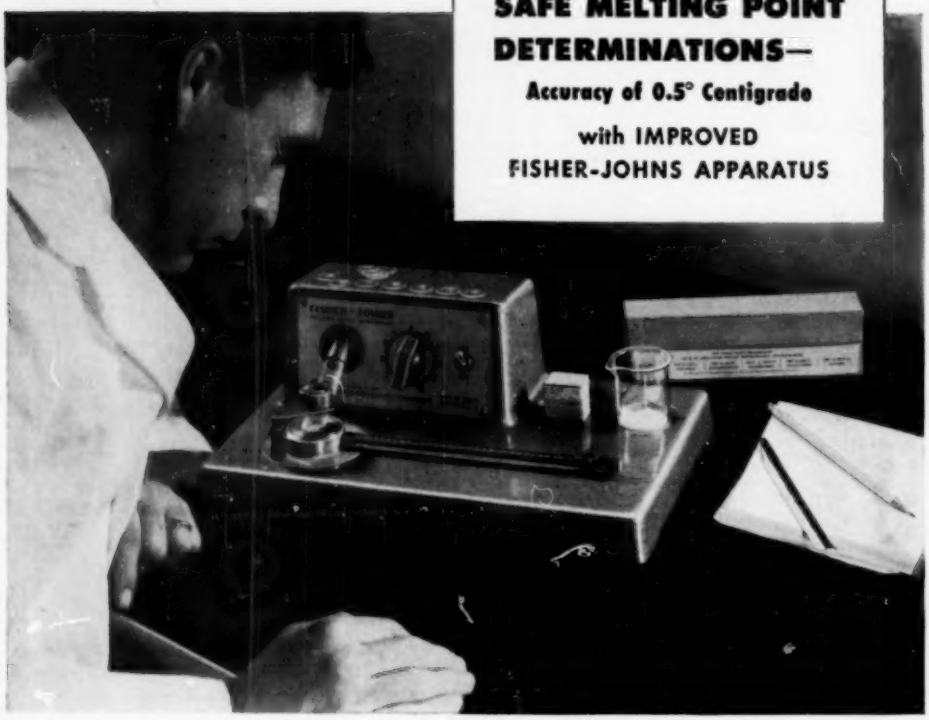

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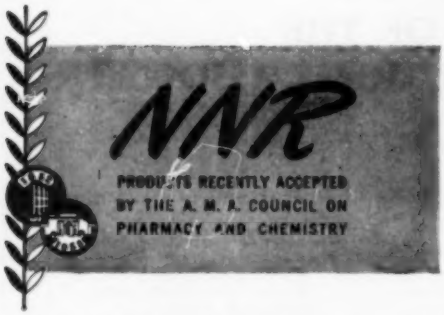
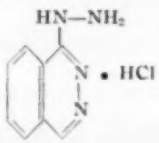


HYDRALAZINE HYDROCHLORIDE.—Apresoline Hydrochloride (Ciba). —$C_8H_8N_4.HCl$.—M. W. 196.64.—1-Hydrazinophthalazine hydrochloride.—The structural formula of hydralazine hydrochloride may be represented as follows:



Actions and Uses.—Hydralazine hydrochloride, a derivative of phthalazine, is an antipressor drug that exerts chiefly a central action on the midbrain. It possesses a moderate degree of adrenergic (adrenolytic and sympatholytic) blocking action against the pressor effects of epinephrine and levarterenol. The drug increases blood flow through the kidney, diminishes cerebral vascular tone, and reduces both diastolic and systolic blood pressure. Hydralazine may inhibit the pressor actions of angiotonin (hypertensin), serotonin, pherentasin, and possibly other endogenous factors considered important in causing hypertension. It also inhibits the hormonal-cerebral vasopressor substance that may participate in varying degrees in more than one form of hypertensive disease and that is not affected by more potent adrenergic blocking agents. The capacity to inhibit a pressor substance of cerebral origin may explain the drug's effectiveness in neurogenic hypertension not benefited by extensive lumbodorsal sympathectomy.

Hydralazine helps control essential and early malignant hypertension. Its efficacy is often greater in acute, severer, nonterminal phases of these disorders. Worth-while results may be expected in the toxemias of pregnancy. Preliminary studies indicate some beneficial effects in acute glomerulonephritis. Hydralazine is thus a useful adjunct in the control of diverse forms of hypertension, with due consideration to the environmental, dietary, and psychic factors involved.

Although true tolerance to the drug has not been demonstrated, blood pressure may rise occasionally during treatment. When this occurs, it may be advisable to discontinue therapy for a week, then resume it with small doses as for initial treatment.

Because the toxicity of hydralazine is low, serious untoward effects are seldom encountered. Studies on experimental animals have not revealed evidence of chronic toxic effects on the tissues. Clinically, postural hypotension and circulatory collapse may precede a fall in blood pressure, but severe reactions of this kind are relatively rare.

The physician must be thoroughly familiar with the characteristics of hydralazine before prescribing or administering the drug. Only with such understanding can the maximal benefit consistent with minimal untoward effects be fully realized. Use of the drug in conjunction with other potent hypotensive drugs should not be attempted until there is further evidence by which to weigh the possible usefulness of combined therapy against its potential dangers.

Caution is advised in the prolonged administration of large doses as they have been reported to produce, in some patients, an apparently toxic response resembling either early rheumatoid arthritis, or, what is regarded as a severer phase of the same syndrome, acute systemic lupus erythematosus. The milder rheumatic phase usually disappears when the drug is withdrawn or the dosage reduced. The severe erythematous form has been controlled with cortisone and corticotropin.

Dosage.—Hydralazine hydrochloride is usually administered orally but may be injected parenterally (intramuscularly or intravenously) when the drug cannot be given by mouth. By either route, the dosage must be individualized in accordance with the response of the patient.

In the ambulatory patient, therapy should be initiated by the oral route and the patient carefully instructed by the physician concerning the subjective effects that are produced. Headache and/or palpitation usually are experienced within twelve to twenty-four hours following the initial dose. These symptoms usually disappear spontaneously, with no change in dosage, within seven to ten days after starting treatment.

The initial dose for moderate to severe hypertension should be 10 mg., given four times daily, after each meal and at bedtime, to make a total daily dose of 40 mg. Individual doses should be spaced equally and the total of 40 mg. per day continued for the first two to four days, unless contraindicated because of severe or distressing side effects. The dose may be increased to 25 mg. four times daily for the balance of the first week. During the second week, the dose may be increased to 50 mg. four times daily (total daily dose of 200 mg.). If side effects are absent or minimal and the blood pressure can be reduced to a more desirable level the single dose may be augmented by 10- or 25-mg. increments every five to seven days. Most patients obtain maximal benefit from the schedule of 100 mg. four times daily (total daily dose of 400 mg.); however, some patients are best stabilized with as little as 100 mg. per day in divided doses. Others may tolerate as much as 600 mg. per day. If tolerance develops, the drug should be withdrawn for one week and then administration resumed at the lowest effective level.

In hospitalized patients with severe types of hypertensive disease, therapy is also initiated by the oral route at a dose of 25 mg. every six hours for the first twenty-four hours. Thereafter, individual doses are increased by 25 mg. daily until the desired effect is obtained. Intervals between individual doses should be equal and no less than four hours apart. The same management of side effects and

(Continued on page IV)

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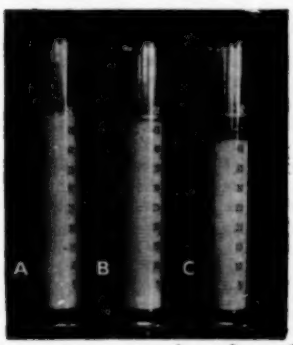

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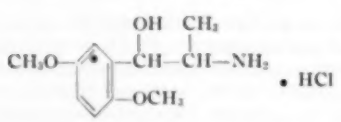
tolerance for the ambulatory patient applies to the hospitalized patient.

Parenteral injection may be used to initiate therapy in hospitalized patients with severe hypertension who are unable to take the drug orally. The usual parenteral dose is 20 to 40 mg. intravenously or intramuscularly, every four to six hours depending upon the patient's response. In patients with extensive renal damage, a smaller initial dose may be indicated. The blood pressure, which should be checked frequently to reveal any precipitous drop, will act as a guide to individual dosage. Pressure may begin to decline within two or three minutes after injection; the average maximal fall occurs within ten to eighty minutes. When the measured fall is not as large as desired, subsequent individual doses may be increased gradually, provided absorption of the initial dose has not been delayed. It is usually possible to shift to oral therapy within twenty-four to forty-eight hours. In cases of preeclampsia or eclampsia, the blood pressure may fall dramatically to normal levels within five to ten minutes, after the intravenous injection of 20 to 40 mg. It is advisable to inject 20 mg. as the initial dose and to follow with another injection if further reduction of blood pressure is desired. The effect may persist 6 to 24 hrs. Additional injections may be given and, as soon as feasible, the patient shifted to oral therapy with the schedule ordinarily followed for hospitalized patients. In hypertensive episodes associated with acute glomerulonephritis, intramuscular injections of 0.25 mg. per kilogram of body weight are usually effective in reducing blood pressure. Injections may be repeated as necessary every four to six hours.

Tests and Standards—

Physical Properties: Hydralazine hydrochloride is a white odorless, crystalline powder, m.p. 270–280° (with decomposition). It is very slightly soluble in ether. The amounts which dissolve in the following solvents to form 100 ml. of solution are 0.2 Gm. in alcohol and 4.4 Gm. in water. The *p*H of a 2% solution is 3.5–4.5.

METHOXAMINE HYDROCHLORIDE.—Vasoxyl Hydrochloride (Burroughs Wellcome).—$C_{11}H_{17}NO_3$.HCl.—M. W. 247.72.—α-(1-Aminoethyl)-2,5-dimethoxybenzyl alcohol.—β-Hydroxy-β-(2,5-dimethoxyphenyl)isopropylamine hydrochloride.—The structural formula of methoxamine hydrochloride may be represented as follows:



Actions and Uses.—Methoxamine hydrochloride is a sympathomimetic amine compound which exhibits the vasopressor action (peripheral vasoconstriction) characteristic of other chemical agents of this class. Unlike the action of most pressor amines, the cardiac rate decreases as the blood pressure increases. This bradycardia which is apparently caused by a carotid sinus reflex mediated by the vagus nerve, is abolished by atropine.

Methoxamine hydrochloride is indicated primarily during surgery to maintain adequately or restore arterial blood pressure, especially in conjunction with spinal anesthesia, which tends to produce a fall in blood pressure. It is also useful as an adjunct in the treatment of hypotension associated with hemorrhage, trauma, and surgery. Its adjunctive use is particularly indicated immediately prior to emergency operations in hypotensive patients who are poor surgical risks. It may be helpful in combating postoperative collapse; however, the drug should not be regarded as a substitute for blood, plasma, or other measures indicated in treating shock.

Like other vasopressor agents, methoxamine hydrochloride is contraindicated in patients with myocardial degeneration or coronary disease. It should be used only with great care, if at all, in patients with cardiovascular disease, hyperthyroidism, or severe hypertension. Caution should be exercised to avoid overdosage resulting in high blood pressure and excessive bradycardia. High dosage occasionally may produce sustained, excessive blood pressure elevations with severe headache. Excessive dosage, especially that given intravenously, may produce headache, pilomotor response, a desire to void, and projectile vomiting.

Dosage.—Methoxamine hydrochloride is administered in solution by either intramuscular or intravenous injection. The latter route should be reserved for emergencies or cases in which the systolic blood pressure falls to 60 mm. Hg or less.

The usual intramuscular dose is 10 to 15 mg. When used to prevent a fall in blood pressure during spinal anesthesia, it is administered intramuscularly at the time of induction and the dose is adjusted in accordance with the level of anesthesia to be employed; 10 mg. may be adequate for operations below the level of the umbilicus, 15 to 20 mg. for those above that level. A second dose should not be given until the previous one has had time to act; usually fifteen minutes is sufficient. A solution of the drug containing 1% procaine hydrochloride may be employed as the prophylactic intramuscular dose immediately prior to spinal anesthesia. From 0.1 to 0.2 cc. of such solution is used to make a skin wheal and produce local anesthesia at the site selected for lumbar puncture. After deeper insertion of the needle, the remainder of the solution needed to provide the pressor dose of the drug is injected intramuscularly. Lumbar puncture is then made through the wheal. In combating hypotension from other causes, the intramuscular dose is similar; for preoperative and postoperative use for moderate hypotension 10 mg. may be adequate.

The usual intravenous dose, reserved for emergencies only, is 5 to 10 mg. administered slowly; however, the latter amount should not be exceeded. Intravenous injection may be accompanied by supplemental intramuscular injection of 10 to 15 mg. to provide a more prolonged effect.

Tests and Standards—

Physical Properties: Methoxamine hydrochloride is a white, bitter, crystalline powder with a very slight odor,

(Continued on page VII)

101st Convention

American Pharmaceutical Association

and

Affiliated and Related Organizations

Hotel Statler
Boston, Mass.
August 22-27, 1954

The next annual meeting of the American Pharmaceutical Association will be held in Boston, Mass., August 22 to 27, 1954.

Members planning to present papers before any Section during this meeting are earnestly requested and strongly urged to notify the appropriate Section secretary of their intention promptly.

Titles and abstracts *(*not to exceed 200 words*)* of papers should be submitted by *July 1, 1954, or preferably earlier,* in order to insure a place on any section program. Send titles and abstracts of papers to the appropriate Section Secretary.

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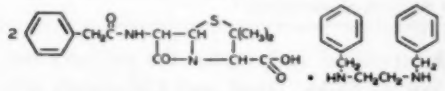
• • • • • • • • • • • • • • • • *from page IV*

m. p. 212–216°. It is freely soluble in water, and very slightly soluble in ether and ethyl acetate. 8.8 Gm. dissolves in alcohol to form 100 ml. of solution. The *p*H of the 2% solution is 4.0–5.0.

BENZATHINE PENICILLIN G.—Bicillin (Wyeth). —N,N′-dibenzylethylenediamine dipenicillin G.— The structural formula of benzathine penicillin G may be represented as follows:



Actions and Uses.—Benzathine penicillin G, a complex salt of penicillin of relatively low solubility in water, exhibits somewhat more prolonged action than more soluble salts of the drug and provides comparable blood levels. Its effective absorption from the gastrointestinal tract is not affected appreciably by food intake; the use of added acid buffers is not required for oral administration. As with other orally administered compounds, it must be dissolved prior to intestinal absorption, but because of the limited solubility of benzathine penicillin G in the stomach, it is not highly susceptible to destruction by gastric juice. It is tasteless and is stable in aqueous suspension for twenty-four months at ordinary room temperatures. Orally it produces effective blood levels when administered in adequate doses at six- to eight-hour intervals. By the intramuscular route, a single injection produces an effective blood level for one to four weeks or longer, depending on the size of the dose.

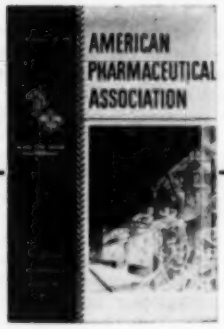
Benzathine penicillin G is indicated for the prevention or treatment of infections susceptible to therapy with penicillin, and, in general, it shares the same indications as other compounds of penicillin G. Because of the infrequency of injection required, parenteral administration is particularly useful for the prevention and treatment of secondary infection following tonsillectomy and tooth extraction for patients with a history of rheumatic fever, in rheumatic heart disease, and whenever prolonged penicillin protection is indicated.

Following oral administration loose stools have been observed in some patients, but in most cases no other signs of toxicity have been reported. Hypersensitivity reactions to this penicillin compound have been infrequently reported.

Dosage.—Benzathine penicillin G is administered orally either as a liquid suspension or as tablets, and parenterally by intramuscular injection as an aqueous suspension.

Orally, a dose of 200,000 units or more every six to eight hours, prescribed in liquid or tablet form, is usually adequate for mild infections. An oral dose of 200,000 units usually provides adequate blood levels for as long as eight hours. Oral doses of 200,000 to 300,000 units every six to eight hours may be necessary to maintain the blood serum concentration above 0.1 unit per cubic centimeter. The dosage for children may be calculated on the basis of 3,000 units per pound (about 6,600 units per kilogram) of body weight, administered every eight hours. In the acute phase of pneumococcic infections (except meningitis) or in streptococcic pneumonia, an initial oral dose of 600,000 units of a potassium penicillin G preparation may be supplemented by the oral administration of 200,000 to 300,000 units every six to eight hours, until temperature has remained normal for at least forty-eight hours. Intramuscular injection of 600,000 units can be used to initiate therapy of pneumococcic and nonhemolytic streptococcic infections without bacteremia, which may be followed by oral administration of 300,000 units every eight hours. If bacteremia is present, the oral dose should be increased to 600,000 units or parenteral therapy should be substituted. An injection of 600,000 units every other day should be used in severe infections. In hemolytic streptococcic infections without bacteremia, 200,000 to 300,000 units orally every six to eight hours for at least seven days is recommended; with bacteremia, an initial injection of 600,000 units should be given, supplemented by oral doses of 200,000 units every eight hours. In micrococcic (staphylococcic) infections without bacteremia, an oral dose of 300,000 units every six to eight hours may be tried, but if ineffective, parenteral therapy should be substituted. When any complication or bacteremia is present in micrococcic infections, parenteral therapy only should be used. Susceptible micrococcic infections may be treated with a dose of 1.2 million units repeated in forty-eight to seventy-two hours if required.

Intramuscular injection of a single dose of 600,000 units is recommended as a preventive measure one day prior to tonsillectomy, tooth extraction, or other minor surgical procedures in patients with a history of rheumatic fever and rheumatic or congenital heart disease. In the prevention of recurrent rheumatic fever, injection of 600,000 units every two weeks or 1.2 million units every four weeks is recommended. This dosage eliminates the streptococcic carrier state in most persons. In acute *beta* hemolytic streptococcic infections, a single intramuscular dose of 600,000 units is usually sufficient. In acute gonorrheal urethritis, a single dose of 300,000 units intramuscularly is adequate to effect a cure in most cases. When gonorrheal urethritis is complicated with a suspected primary lesion of syphilis, it is advisable to treat the patient with either sulfonamides or with streptomycin rather than penicillin until the diagnosis of syphilis can be verified or excluded by repeated darkfield examinations and serologic examination of the blood repeated at monthly intervals for three months. In gonorrheal complications, repeated injections are rarely necessary. In gonorrhea complicated by suspected primary syphilis, an injection of 1.2 million units may be expected to eradicate or abort syphilitic infection.



A Preview of Advertising

In the June 1954 issue of the Practical Pharmacy Edition

(All references to actions and uses are taken from the statements of the producers of the products mentioned.)

▸ Currently featured by **Abbott Laboratories** are their antibiotic, **Erythrocin,** and **Optilets,** multivitamin tablets.

▸ **The American Druggists' Fire Insurance Co.** stresses that its service is "for druggists only."

▸ **Clinitest,** sealed-in-foil reagent tablets, are offered by the **Ames Company** as a "practical, profitable suggestion."

▸ The **Chesebrough Mfg. Co.** emphasizes the dependability of its **Vaseline** sterile petrolatum gauze dressings.

▸ **Hynson, Westcott & Dunning** describes **Lutrexin Tablets** as valuable in the treatment of dysmenorrhea.

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▸ **A. H. Robins, Inc.** recommends **Mephate** "when the doctor's patient doesn't know how to relax."

▸ The **Roerig** announcement stresses **Tetracyn's** profit potential.

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▸ **Crystoserpine** is a new addition to the **Smith-Dorsey** line of Rauwolfia preparations.

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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLIII — *JUNE, 1954* — NUMBER 6 — CONSECUTIVE NO. 12

Biologically Active Nuclear-Substituted Sulfonamides*

I. Chemical and Physical Properties of Some Nuclear-Substituted Sulfanilamido-2-Thiazoles and Their Syntheses

By S. F. QUAN,† T. C. DANIELS, and W. D. KUMLER

The 2-chloro-, 2-methyl-, and 3-methyl-benzene-ring substituted sulfathiazoles were prepared by interaction of the respective acetylated aminobenzene sulfonyl chloride and 2-aminothiazole in ethyl acetate, followed by hydrolysis of the acetyl group and purification. The 3-azobenzene derivatives were prepared by coupling of sulfathiazole with the diazonium salt of the respective anilines. The pK_a of these compounds varied from 5.70 for 2-(3-azo-*p*-nitrobenzene sulfanilamido-) thiazole to 7.30 for 2-(2-methyl sulfanilamido-) thiazole. The acetylation number for the diazo compounds was abnormally high. The acetylated sulfonamides hydrolyzed at approximately the same rate. Attempts to determine the reactivity of the N^4-amino group by acetylation, oxidation with ceric sulfate, and Schiff's base formation were unsuccessful. The absorption spectrum of 3-methyl-substituted compound differs greatly from the parent, sulfathiazole, the 2-methyl- and 2-chloro-sulfathiazole analogs, and the 3-azobenzene derivatives. This difference is attributed to steric effects.

FOLLOWING the epochal discovery of the effectiveness of sulfonamide derivatives in the chemotherapy of bacterial infections (1), thousands of sulfonamide derivatives have been synthesized and studied (2). The compounds thus far studied included few nuclear-substituted sulfonamide derivatives. Northey (3) has suggested that this is likely due, in part, to the greater difficulty of preparation of this type of compound and to the low activity or lack of activity shown by the nuclear-substituted compounds first studied. True, these derivatives are more difficult to prepare, but the synthesis of some is not very different from that of the known sulfonamides. Several nuclear-substituted sulfonamides have been prepared and studied. Their structural composition and chemical analyses are tabulated in Table I. Other chemical and physical measurements were made on the theory that the N^4-amino group is related to the biological action of the compound. The results of the preliminary attempts to determine the reactivity of this amino group by acetylation, hydrolysis of the acetylamides, oxidation with ceric sulfate, and Schiff's base formation are discussed.

Sulfathiazole (ST) can be prepared in a number of ways. The one generally used consists of condensation of *p*-acetoaminobenzene-sulfonyl chloride with 2-aminothiazole in dry pyridine or other suitable solvent. When this procedure was contemplated for preparation of nuclear-substituted ST, the major synthetic problem became

* Received October 12, 1953, from the University of California College of Pharmacy, San Francisco 22, Calif.

This paper is abstracted from the dissertation submitted by S. F. Quan to the Graduate Division, University of California, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Pharmaceutical Chemistry.

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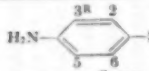
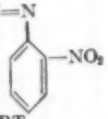
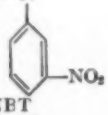
The authors wish to express their thanks to Professor Louis A. Strait for his consultation on the interpretation of ultraviolet absorption spectra.

TABLE I.—COMPOSITION, pK_a, AND MELTING RANGE OF COMPOUNDS STUDIED



Compound		Molecular Weight			Melting Range, °C. (Corr.)	Carbon, %		Hydrogen, %		Nitrogen, %	
R Symbol	Composition	Calcd.	Nitrosation	pK_a		Calcd.	Found	Calcd.	Found	Calcd.	Found
2,—CH_3	$C_{10}H_{11}N_3O_2S_2$	269.3	274	7.30	215	44.59	44.38	4.12	4.13	15.6	15.41
2-MT			266				44.26		4.22		
3,—CH_3	$C_{10}H_{11}N_3O_2S_2$	269.3	267	7.00	196.5	44.59	44.60	4.12	4.22	15.6	15.61
3-MT			262				44.59		4.08		
2,-Cl	$C_9H_8N_3O_2ClS_2$	289.8	293	6.95	223.5	37.4	37.52	2.76	2.79	14.5	14.3
2-CT			286				37.62		2.86		14.4
3,—N=N— 	$C_{15}H_{12}N_6O_4S_2$	404.4	...	6.45	Ca. 190 dec.	44.5	44.59	2.97	3.08	19.9	...
3-*o*-NBT							44.42		2.93		
3,—N=N— 	$C_{15}H_{12}N_6O_4S_2$	404.4	...	6.25	Ca. 160 dec.	44.5	44.64	2.97	3.09	19.9	...
3-*m*-NBT							44.64		2.91		
3,—N=N—	$C_{15}H_{12}N_6O_4S_2$	404.4	...	5.70	Ca. 200 dec.	44.5	...	2.97	...	19.9	19.6
3-*p*-NBT											19.6
3,—N=N—	$C_{15}H_{12}N_6O_2S_2$	359.4	...	5.85	Ca. 180 dec.	50.2	49.95	3.64	3.69	19.7	...
3-BT							49.68		3.66		

the preparation of the appropriately substituted sulfonyl chloride.

Chlorosulfonation was carried out according to the method of Smiles and Stewart given in *Organic Syntheses*. The crystallization procedure, however, was found to be impractical and could not be applied to some of the ring-substituted sulfonyl chlorides. A modification for purification proposed by Pence and Winter (4) requires centrifugation in the cold and evaporation of large volumes of ethereal solution, both operations being inconvenient. For this study, instead of purification or crystallization, the reaction product containing an excess of chlorosulfonic acid was stored in a glass-stoppered bottle. When the acid chloride was needed, a weighed portion of the chlorosulfonic acid solution was poured over cracked ice, the crude product was collected, washed free of acid, and dried. This procedure was used and found to be satisfactory and convenient for preparing the 2-chloro-, 2-methyl-, and the 3-methyl-4-acetaminobenzene sulfonyl chlorides.

The best solvent found for condensing the acid chloride with the 2-aminothiazole was ethyl acetate. The sulfonamide product could be isolated more easily and the quality was better than when pyridine, acetone, dioxane, ether, or benzene was used.

The diazo dyes of sulfathiazole were prepared according to the procedure of Sah and Oneto (5) in good yields.

The melting range and the pK_a in water of the new compounds are presented in Table I.

The pK_a was determined by dissolving one m*M* of the sulfonamide in 20 cc. of ethanol and 20 cc. of aqueous 0.1 *N* NaOH, and then titrating the solution with 0.2 *N* HCl in 50% ethanol solution using a glass electrode. The results are shown in Fig. 1. To convert these results from the alcohol-water system to the conventional water system, in which the compounds are slightly soluble, the curve shown in Fig. 2 was plotted. The pK_a's for the nuclear-substituted compounds were then interpolated. This procedure was used by Bell and Roblin (6) and its physical significance has been substantiated. Furthermore, the values obtained for sulfanilamide (SAN), sulfadiazine (SD), and ST, as shown in Fig. 2, are similar to those obtained by Bell and Roblin (6).

Some unexpected high acetylation numbers resulted with the reaction of acetic anhydride and the newly prepared compounds in pyridine. ST and 2-(2-methylsulfanilamido-)thiazole (2-MT) each yielded one equivalent, 2-(2-chlorosulfanilamido-)thiazole (2-CT), 2, but sulfanilic acid (SAA), 6. The values for the same reaction with the diazo dyes are still higher. 2-(3-Azo-*o*-nitrobenzenesulfanilamido-)thiazole (3-*o*-NBT) gave 7; 2-(3-azobenzenesulfanilamido-)thiazole (3-BT), 15; and 2-(3-azo-*m*-nitrobenzenesulfanilamido-)thiazole (3-*m*-NBT), 18 equivalents. In part, the intermediate values are probably due to acetylation at all possible positions on the nitrogen atoms. This type of acetylation is similar to that reported by Seikel (7) in her study of the 3,5-dichlorosulfanilamide. In addition there may also have been condensation of the acetylating agent. The higher acetylation numbers, however, suggest excessive condensation of the acetic anhydride.

All of the acetylated sulfonamides hydrolyzed at about the same rate. This is to be expected since the rate of hydrolysis of esters and amides in general depends on the acid moiety.

The results of an attempt to follow the rate of acetylation by using the diazotization reaction could not be correlated with the reactivity of the amino group. With some compounds the diazotization proceeded slowly and the results become unreliable.

When the rate of oxidation with ceric sulfate solution was followed by diazotization, similar difficulty in slow diazotization was again encountered. The rates for different compounds could not be compared. The use of barium diphenylamine sulfonate as an oxidation-reduction indicator to follow the rate of oxidation instead of diazotization did not lead to satisfactory results.

The rate of Schiff's base formation with the sulfonamides was not studied because Werner's procedure (8) for estimating the amount of SAN in biological fluids could not be applied.

Introduction of a nuclear substituent into a resonating system such as SAN changes its resonance. This change is detected in the ultraviolet absorption spectra of the compounds. In Figs. 3 and 4 it can be seen that the changes in the extinction coefficient and in the wave length of the absorption maxima resulting from substitution in the benzene nucleus of ST is small, with the exception of the compound substituted by a methyl group in the 3-position (3 MT). The small changes in absorption, noted for 2-CT, may be adequately accounted for by the change in the distribution of electrical charges in the molecule and, as in the diazo dyes, by the increased molecular weight. The pronounced change of the spectrum of 3 MT is due to the steric effect of the methyl group being *ortho* to the amino group (9). Such a methyl group tends

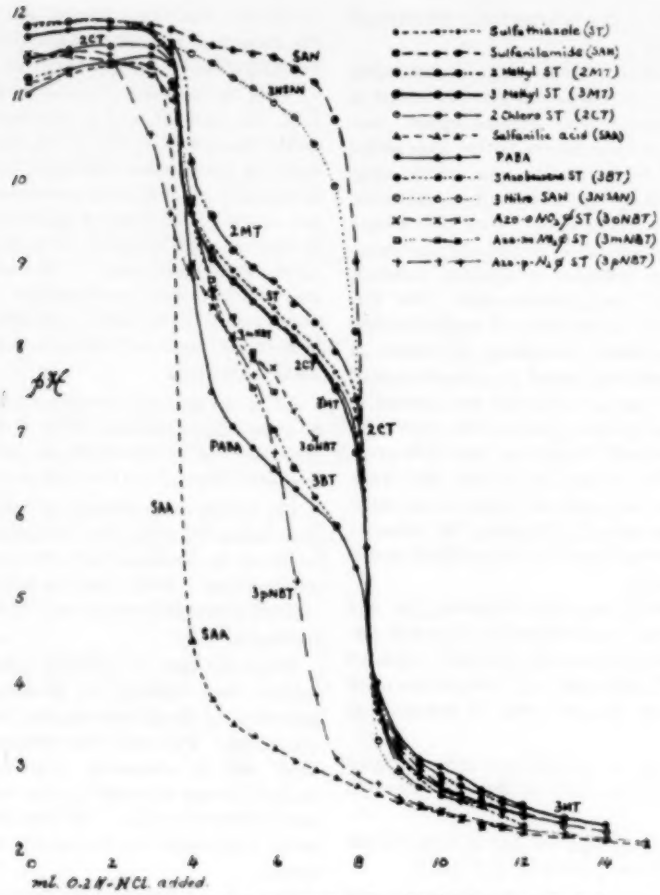


Fig. 1.—Acid titration curves of some sulfas in aqueous-alcohol.

to interfere with the coplanar configuration of the amino group (10) and thereby reduces considerably the contribution to the resonance of the molecule by the form:

EXPERIMENTAL

2-Chloro-4-acetaminobenzene Sulfonyl Chloride.—In a 3-neck, 500-cc. flask, equipped with stirrer and thermometer, was placed 400 Gm. (3.76 mole, 250 cc.) of chlorosulfonic acid (tech.). The liquid was stirred and cooled to 8° in an ice bath and then 115 Gm. (0.67 mole) of *m*-chloroacetanilide was added in small portions and at such a rate that the temperature did not rise above 15°. The ice bath was replaced by a "Glas-col" electric heating mantle, and the solution was slowly heated to 43° (in two hours), then rapidly to 70° (during the next ten minutes) and kept at that temperature, stirring continuously for the next six hours. The heating mantle was removed and the dark-brown solution was allowed to cool. It was then poured slowly with mixing and stirring into 3 L. of cracked ice (no water added). A fine light precipitate formed, which was filtered and changed slowly to a sticky, tan mass. The product was taken up in 500 cc. of acetone and again filtered. The filtrate, when it was poured into 1,500 cc. of ice and water, gave a semisolid, oily precipitate, which did not change in consistency even after sitting in the refrigerator for two hours. The aqueous supernatant liquid was discarded and the oily residue was dissolved in 300 cc. of ethyl acetate. In order to remove the residual water, the ethyl acetate solution was treated first with sodium chloride and then dried with anhydrous sodium sulfate. Yield: *ca.* 50% or less.

2-Methyl-4-acetaminobenzene Sulfonyl Chloride.—This was prepared in the same manner as the 2-chloro-4-acetaminobenzene sulfonyl chloride. To complete the reaction, it was necessary to heat at 60° for nine hours. The oily product obtained after washing with ice water was dried in ethyl ether solution. Yield: *ca.* 50%.

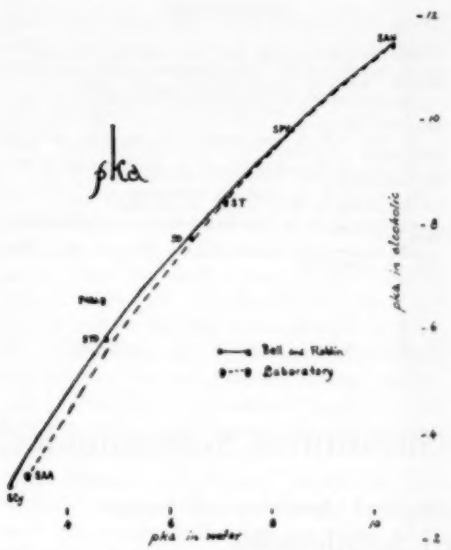


Fig. 2.—Relationship of aqueous and aqueous-alcoholic pK_a.

3-Methyl-4-acetaminobenzene Sulfonyl Chloride.—This product was prepared in the manner described above. To complete the reaction it was necessary to heat to 60° and hold at that temperature for five or six hours. The crude product obtained by pouring the chlorosulfonic acid solution on ice was dissolved in acetone, filtered, and reprecipitated by pouring the acetone solution into a large volume of water. The precipitate was collected and dried in a desiccator overnight. Yield: 70–85%.

2-(2-Chlorosulfanilamido)-thiazole.—In a 3-neck, 500-cc., round-bottom flask, equipped with a mechanical stirrer, dropping funnel, and thermometer, was placed 40 Gm. (0.4 mole) of 2-aminothiazole, 50 cc. (45 Gm.) of ethyl acetate, and 20 cc. of dry pyridine. The mixture was cooled (ice-salt bath) with stirring to a temperature of 4° when 300 cc. of dried ethyl acetate solution of the 2-chloro-4-acetaminobenzene sulfonyl chloride (equivalent to approximately 0.3 mole) was slowly added with vigorous stirring. After all of the acid chloride solution had been added, 10 Gm. (0.15 mole) of anhydrous sodium acetate was introduced into the reaction mixture. Stirring of the mixture was continued for fourteen to eighteen hours at room temperature. The ethyl acetate was removed by distillation under reduced pressure. The sticky brown residue was stirred with 150 cc. of cold water, whereupon it changed to a fine, tan, granular solid. The precipitate was filtered and washed with water. It was then suspended in 500 cc. of 2 *N* NaOH and refluxed for thirty minutes to insure complete hydrolysis of the acetylated compound. The solution was filtered while hot and the filtrate made acid with 4 *N* HCl (500 cc.). This solution was decolorized twice with 4 Gm. of Norite®. The filtrate was neutralized with a saturated solution of sodium hydroxide. On cooling a light tan granular solid was formed and collected. When dried, the product weighed 35 Gm. Yield: approximately 20%. The crude product was purified by repeated crystallization from 95% ethanol solution. White flakes were obtained; m. p. 223.5°

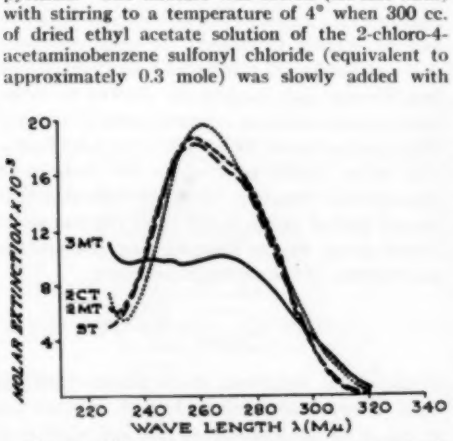


Fig. 3.—Ultraviolet absorption spectra of simple nuclear-substituted sulfathiazoles.

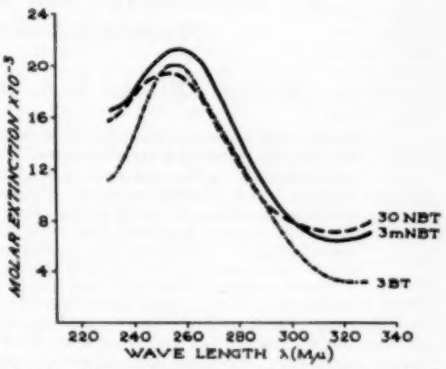


Fig. 4.—Absorption spectra of azobenzene substituted into sulfathiazole nucleus.

2-(2-Methyl Sulfanilamido-)thiazole.—This compound was prepared and purified in the same manner as the 2-chloro compound described above. Yield: *ca.* 40%. White crystals were obtained from 95% ethanol solution; m. p. 215°.

2-(3-Methyl Sulfanilamido-)thiazole.—This product was prepared in the same manner as described above, but was hydrolyzed with 10% HCl and recrystallized from a 10% acetic acid solution. Yield: 67%, m. p. 196.5°.

2-(3-Azo-*p*-nitro-benzene Sulfanilamido-)thiazole.—To a solution of 13.8 Gm. (0.1 mole) of *p*-nitro-aniline in 50 cc. (0.87 mole) of glacial acetic acid and 25 cc. (0.28 mole) of concentrated HCl, contained in a 600-cc. beaker immersed in an ice bath, was added 100 Gm. of cracked ice and 6.9 Gm. (0.11 mole) of sodium nitrite dissolved in 15 cc. of water. The slight excess of nitrous acid was decomposed with urea. Then a solution prepared by dissolving 27 Gm. (0.11 mole) of sulfathiazole and 8 Gm. (0.2 mole) of sodium hydroxide in 150 cc. of water was poured with vigorous stirring into the iced solution of the diazonium compound. A thick, dark brown precipitate formed almost im-

mediately. The cold mixture was stirred for ten minutes and then heated on a steam bath to a temperature of 65–70° for one hour. The dye was collected by filtration and washed with water. It was further purified by dissolving in a solution of sodium hydroxide, precipitating with a dilute solution of hydrochloric acid, and washing with water.

The yields for this and other ST azo dyes were almost quantitative.

The color of the azo dyes in this study varied from a deep yellow to brown. In alkaline solution they were deep red to purple.

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Biologically Active Nuclear-Substituted Sulfonamides*

II. Bacteriostatic and Biological Activity of Some Nuclear-Substituted Sulfathiazoles

By S. F. QUAN,† T. C. DANIELS,‡ and K. F. MEYER§

Seven new nuclear-substituted sulfathiazole derivatives were tested *in vitro* against *Pasteurella pestis* and *Bacillus subtilis*, and were compared with sulfathiazole and sulfanilamide in the presence and absence of *para*-aminobenzoic acid (PAB) by using a broth dilution method. Each of the nuclear-substituted sulfonamides was at least as active or more active than the parent compound, sulfathiazole. In *in vivo* experiments, however, they were not superior to sulfathiazole in the therapy of either plague, staphylococcus, or streptococcus infection in the mouse. Generally, these compounds are more toxic than sulfathiazole. Since the antibacterial activity of these nuclear-substituted derivatives is antagonized by *p*-aminobenzoic acid, they conform to the accepted definition of a true sulfanilamide-type compound. On the other hand, two thiophene sulfonamides tested were active antibacterial agents, but they were not inactivated by PAB. The activity of these nuclear-substituted derivatives modifies some of the current concepts with regard to the relationship between the sulfanilamide structure and antibacterial activity.

THE LITERATURE on the sulfonamide compounds gives insufficient information on the antibacterial activity of nuclear-substituted sulfonamides to justify the conclusion that such compounds are inactive (1, 2). Nevertheless, none are reported to be active. Furthermore, most of the published data indicate that nuclear substitution in sulfanilamide and its derivatives drastically reduces or completely abolishes the bacteriostatic activity.

* Received October 12, 1953, from the University of California College of Pharmacy, San Francisco 22, Calif.
This paper is abstracted from the dissertation submitted by S. F. Quan to the Graduate Division, University of California, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Pharmaceutical Chemistry.
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§ Director, George Williams Hooper Foundation for Medical Research, University of California Medical Center, San Francisco 22, Calif.

The assumption that nuclear-substituted sulfonamides are generally inactive led to speculation and hypotheses to account for their inertness. Bell and Roblin (3) attributed it to steric effects, and Kumler and Daniels (4) pointed to interference with resonance. Incompatibility of steric effects in the former theory will be explained later. The latter theory referred to the findings of Ingham and Hampson (5), which indicated that even a methyl group in the *ortho* position to an amino group in the benzene ring reduces the contribution of the coplanar resonance

forms. This reduction in resonance that has been observed in compounds with substitution in the 3- or 3,5-positions is real and significant but, judging from the work of Quan, *et al.* (6),

such steric hindrance is not present in sulfonamide compounds with 2 or 2,6 substituents. Therefore, all ring-substituted sulfanilamides cannot be considered either inactive or always to have the resonance of the *para*-aminobenzene sulfonamide groups sterically impeded.

The few nuclear-substituted compounds that have been studied for antibacterial action were tested before it was known that *para*-aminobenzoic acid, (PAB), inhibits the action of sulfonamides (7). The PAB effect not only defines sulfa drugs sharply, but also affords a more precise method for assaying of antibacterial activity. It appears likely that if this method had been available for the early studies on the relationship of structure to antibacterial action, the activity of various nuclear-substituted compounds would have been recognized.

The chemical and physical property of these sulfanilamido - nuclear - substituted - 2 - thiazoles have been presented in a previous paper. This report presents experimental evidence that they are biologically active sulfonamides. The toxicity and the chemotherapeutic effects of these compounds will also be described.

EXPERIMENTAL

Bacteriostasis of Pasteurella Pestis.—The broth used in all these experiments was hormone broth (8).

Stock solutions of the 5.0×10^{-5} *M* sulfonamides were prepared in sterile broth and the addition of *N* NaOH was added as necessary. For the testing of *Pasteurella pestis*, 1 cc. of the 5.0×10^{-5} *M* solution was added to the first tube of a series of 10 small (13 x 100 mm.) sterile test tubes, each already containing 1 cc. of sterile broth. From the first tube 1 cc. was transferred to the second tube, and from the second tube, 1 cc. to the third tube, etc., to make a twofold serial dilution. To each tube of the series was then added 1 cc. of 10^{-2} broth dilution of a 24-hour 37° broth growth suspension of *P. pestis*, strain A1122. The tubes were shaken and then incubated at 37°. After 24, 48, and 120 hours the degree of growth was read as indicated by the turbidity of the broth in the tubes and then one standard 3-mm. loopful was removed from each tube and streaked onto a section of a blood agar plate. The agar plates were incubated and later read for growth. The results of this experiment are shown in part in Table I.

The growth of other bacteria was similarly tested.

Bacteriostasis of Bacillus Subtilis with and without PAB.—To compare the effect of PAB on the sulfonamide activity, two series of the same dilutions of each compound were prepared as above. Since *P. pestis* does not respond to PAB, *Bacillus subtilis* was used as the test organism. It was grown eighteen hours in broth at 37°, then was diluted 1:10,000 with plain broth and with broth containing 10^{-4} *M* PAB. These dilute bacterial suspensions were inoculated into broth which contained different sulfonamides in varying concentrations and then incubated at 37°. The results of this experiment are in part shown in Table II.

Toxicity.—Of each compound a 5-cc. solution of 0.20 millimole (m*M*) was made with 0.5 cc. of *N* NaOH and 4.5 cc. of sterile physiologic sodium chloride solution. Each solution was injected intravenously into the lateral tail vein of albino laboratory (Swiss) mice in doses of 0.5 cc. per 20 Gm. body weight. The LD_{50}, shown in Table III, was calculated from the number of deaths within twenty-four hours after injection of the drug.

Chemotherapy of Plague.—Sixty albino (Swiss) mice weighing approximately 20 Gm. were inoculated subcutaneously with 0.2 cc. of a 10^{-4} dilution of a successive day transfer of a broth of virulent plague (Shasta strain) culture into the lower abdominal region between the right inguinal and the central abdominal lines. The infection dose was between 2,000 and 4,000 organisms per mouse. The mice were deprived of drinking water after inoculation. On the 48th hour after infection, drugged water containing 10^{-3} *M* of sulfonamide was given to the mice. These solutions were the mice's only source of drinking water for the next ten days. The control mice drank plain water throughout.

Part of the protocol of the plague experiment is given in Table III.

TABLE I.—GROWTH OF *Pasteurella Pestis* IN SULFONAMIDE BROTH[a]

Symbol	Sulfonamide (H_2N—⟨⟩—SO_2—NHR)	Growth in 24 Hr. Dilutions of 5×10^{-5} *M* 1:4	1:8	1:16	Growth in 120 Hr. Dilutions of 5×10^{-5} *M* 1:4	1:8	1:16
SAN	Sulfanilamide	+(4+)	+(4+)	+(4+)	4+(4+)	4+(4+)	4+(4+)
ST	Sulfathiazole	+(4+)	+(4+)	+(4+)	3+(4+)	4+(4+)	4+(4+)
2MT	2′-Methyl sulfathiazole	+(4+)	+(4+)	+(4+)	3+(4+)	4+(4+)	4+(4+)
3MT	3′-Methyl sulfathiazole	+(4+)	+(4+)	+(4+)	++(3+)	4+(4+)	4+(4+)
2CT	2′-Chlorosulfathiazole	−(4+)	+(4+)	+(4+)	−(0)	++(4+)	4+(4+)
3BT	3′-Azobenzene sulfathiazole	−(0)	−(0)	−(500)	−(0)	−(0)	−(0)
3*o*NBT	2-(3-Azo-*ortho*-nitrobenzenesulfanilamido)-thiazole	−(++)	−(3+)	+(4+)	−(0)	±(0)	++(150)
3*m*NBT	2-(3-Azo-*meta*-nitrobenzenesulfanilamido)-thiazole	+(4+)	+(4+)	+(4+)	+(0)	+(0)	++(0)

[a] Concentration of *P. pestis* *ca.* 10^8/cc. Degree of turbidity indicated by 1 to 4 plus signs.—Degree of confluency of growth or number of colonies occurring on the section of the blood agar plate streak with a 3-mm. standard loop in paren theses.

TABLE II.—EFFECT OF SULFONAMIDES ON *Bacillus subtilis* WITH AND WITHOUT PAB[a]

Compound	S[b]	Turbidity of Growth After 12 Hr. at 37° C. — With PAB — Reciprocal of the Dilution: 2	4	8	16	32	∞	Without PAB — Reciprocal of the Dilution: 2	4	8	16	32	∞
SAN	1/2	3[c]	4	4	4	4	4	0	0	0	+	+	4
ST	1/2	2	3	4	4	4	4	+	+	0	0	0	4
2MT	1/2	2	3	3	4	4	4	+	+	±	0	±	4
3MT	1/2	+	2	3	4	4	4	+	+	+	+	+	4
2CT	1/2	+	2	2	2	3	4	+	+	±	±	±	4
3BT	1/20	0	0	+	2	3	4	0	0	+	+	2	4
3*o*NBT	1/20	+	2	4	4	4	4	±	+	+	2	2	4
3*p*NBT	1/20	0	+	2	3	3	4	0	±	+	+	+	4
NOP[d]	1/4	0	0	±	3	3	4	0	0	±	3	3	4
AP[e]	1/2	0	3	4	4	4	4	0	3	4	4	4	4

[a] Organism: *Bacillus subtilis* (Merck 3R9675): 18-hr. growth in broth at 37° C.—Dilution of culture: 10^{-4} with hormone broth.—Concentration of stock chemical solutions: 5×10^{-4} *M*.—Concentration of PAB: 5×10^{-5} *M*.
[b] Dilution of the stock solution used in the first tube of the series. Symbols same as Table I.
[c] Degree of bacterial growth indicated by 1 to 4 plus signs.
[d] 2-Nitro-thiophene-4-sulfonamide.
[e] 2-Amino-thiophene-4-sulfonamide.

Some of these compounds were tested also on *Staphylococcus aureus* and *Streptococcus pyogenes* infections. None were found to be better than sulfathiazole (ST).

DISCUSSION

In the early studies of the biological activity of SAN and its derivatives, *in vivo* procedures were used almost invariably (9–11). Although the animal test is usually the more significant in determining the clinical usefulness of any therapeutic agent, it is more difficult to control and generally less sensitive than most *in vitro* methods. In this case because the fundamental point in question concerns the antibacterial potency, a precise *in vitro* procedure is invaluable. Antagonism of the bacteriostatic activity of sulfanilamide, (SAN), by PAB provides an accurate *in vitro* technique. With this technique the presence of antibacterial activity, which could be antagonized by PAB, in nuclear-substituted sulfanilamido-2-thiazoles has been definitely demonstrated. Recently, other investigators (12, 13) have also found this type of sulfonamide biologically active.

Substitution in the ring of SAN must contribute to the charge on the sulfur atom. Nevertheless, the original hypothesis advanced to correlate the negativity of the —SO_2— group with the antibacterial activity of a sulfanilamide compound discounts the benzene-ring substituted compounds because of their inactivity (3). Now that these compounds have been proved active and unhindered, this hypothesis should be re-evaluated, if possible, to include the benzene-ring substituted compounds.

Ingham and Hampson (5) demonstrated that a methyl group substituted in the *ortho* position to an aromatic amine significantly lowered the resonance. The ultraviolet spectrum of 2-(3-methyl sulfanilamido) thiazole (3MT), has been shown in a previous publication to demonstrate reduced resonance (6). If the biological activity is related to the coplanar resonance of an aminobenzene sulfonamide, 3MT should show lower bacteriostatic activity than the unsubstituted sulfonamide, ST. This decrease in activity was not observed. Furthermore, the azo compounds derived by coupling ST with diazotized anilines, neither enhance nor contribute to the resonance of the coplanar forms of aminobenzene sulfonamides. Yet, these same sulfonamide azo dyes are many times more active bacteriostatically than the unmodified parent compound, ST. Therefore, the theory that the bacteriostatic activity is directly related to the resonance and the contribution of the coplanar forms,

etc., and suggests that any interference to the coplanarity, or reduction of the contribution of the

TABLE III.—SOME SULFONAMIDES: TOXICITY AND THERAPY OF PLAGUE MICE[a]

Drug	LD_{50} Toxicity — Acid mM/Kg.	Sod. Salt, Gm./Kg.	Number Mice Treated	Survivors — Number of the Day after Infection: 4	5	6	7	8	9	10	12	14
SAN	3.6	0.62	6	4	3	2	2	2	1	1	0	0
3MT	*ca.* 2.3	0.62	6	6	5	4	4	3	2	0	0	0
2MT	*ca.* 1.5	0.39	6	6	5	5	5	3	3	1	1	1
2CT	2.2	0.64	6	6	5	4	4	4	3	3	1	0
3BT	0.9	0.32	6	6	6	6	6	5	5	4	2	1
3*o*NBT	*ca.* 0.8	0.32	6	6	5	4	3	3	3	2	1	1
3*m*NBT	0.7	0.28	6	5	4	3	2	2	2	1	1	1
3*p*NBT	*ca.* 0.7	0.28	0	...	...	...	...	...	...	...	...	...
ST	2.8	0.70	6	6	6	6	4	4	2	1	1	1
SD	4.4	1.10	0	...	...	...	...	...	...	...	...	...
H_2O	...	...	10	8	6	4	3	3	3	3	3	3

[a] Symbols not in Table I: SD = sulfadiazone.—3-*p*-NBT = 2-(3-azo-*para*-nitrobenzene-sulfanilamido)thiazole.

resonance forms should lead to a lowered or reduced biological activity as compared to a compound without these steric effects, is not supported by these findings.

The activity of these nuclear-substituted sulfonamides might have been anticipated from the work of Wyss, Rubin, and Strandskov (14) who observed that substitution of halogens into the benzene ring of PAB varied its ability to antagonize SAN. Derivatives of PAB were studied that increased, decreased, or abolished the antagonistic activity for SAN. Therefore, if SAN acts biologically by competitive inhibition of PAB, as proposed by Woods (7), then it would be logical to assume that similar changes in biological activity should occur for the analogous benzene-ring substituted SAN derivatives This hypothesis is being tested. However, even without the foregoing interpretation, there is now direct evidence to show that the bacteriostatic activity of SAN may be enhanced or suppressed by nuclear substitution.

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Some New Derivatives of Gentisic Acid*

I. Preparation and Proof of Structure of Some New Derivatives of Gentisic Acid

By W. E. MOORE,† F. W. BOPE,‡ and B. V. CHRISTENSEN§

The synthesis of seven new derivatives of gentisic acid is discussed and their analyses and proof of structure are presented. Four derivatives of gentisic acid with amino alcohols were prepared by two type reactions. Three derivatives of gentisic acid with other carboxylic acids were prepared by one type reaction.

THE WORK undertaken by the authors was the extension of the work begun by Nash, *et al.* (1), in the field of amino alcohol and carboxylic-acid esters of gentisic acid. The seven recently prepared derivatives of gentisic acid round out and extend the series of homologous esters of gentisic acid begun by Nash, *et al.* The activity of these seven new derivatives in four selected pharmacological screening procedures is described elsewhere (2).

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EXPERIMENTAL

Preparation of Esters of Gentisic Acid with Other Carboxylic Acids.—Esters of gentisic acid with other carboxylic acids were prepared by the reaction of aryl and acyl chlorides with gentisic acid in an inert solvent in the presence of anhydrous zinc chloride. The reaction consisted of the removal of the elements of HCl between an aliphatic or aromatic RCOCl group and the phenolic OH group located in the 5-position of gentisic acid. Equal molar quantities of the acid chloride and gentisic acid were dissolved in anhydrous ether containing 1 Gm. of freshly fused zinc chloride. The reaction mixture was refluxed 1–4 hours and the ether removed under vacuum. Fresh solvent was added and refluxing repeated for 1–3 hours.

5,5′-Digentisyl Phthalate.—After the refluxing period, the ethereal solution was filtered from a white amorphous precipitate corresponding to 31.7% yield. The product was recrystallized from ethylacetate and petroleum ether (1:1) and acetone and ethylene dichloride (1:1) to give white crystalline plates, m. p. 252–253°.

5-Diphenylacetyl Gentisic Acid.—After the refluxing period, the ether was removed under vacuum and the residue recrystallized from hot 60% ethanol to give a white amorphous powder corresponding to 67.7% yield. The white amorphous material was recrystallized three times from hot ethylene di-

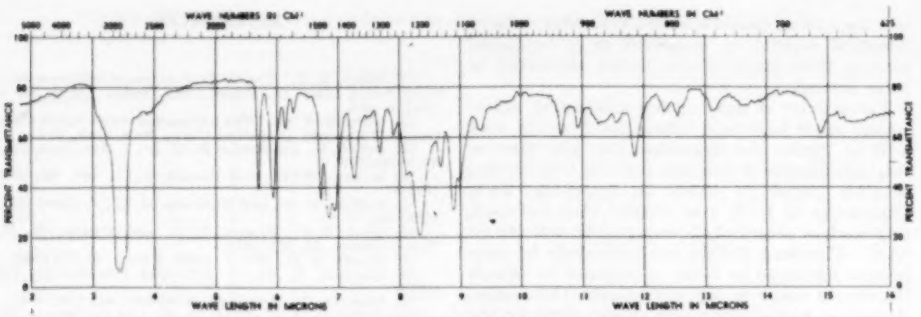


Fig. 1.—Infrared absorption spectrograph of 5-diethylacetyl gentisic acid in Nujol®.

chloride to give a white crystalline compound, m. p. 200.5–201.5°.

5-Diethylacetyl Gentisic Acid.—After the refluxing period, the ether was removed under vacuum. The residue was washed with hot water and the water decanted from the resulting oil. The oil was dissolved in wet ether and the ethereal solution separated from a few ml. of water. The ether was evaporated to dryness to give a white crystalline residue corresponding to 50% yield. The product was washed four times with water, sucking the product dry between washings. Recrystallization from hot Skellysolve® B gave gray-white crystalline needles, m. p. 111.2–113.2°.

Preparation of Derivatives of Gentisic Acid with Amino Alcohols.—The derivatives of gentisic acid with amino alcohols were prepared by two procedures:

A.—2-Diethylaminoethyl-5-ethoxy salicylate hydrochloride was prepared according to the procedure described by Nash, *et al.* (1), by the reaction of 5-ethoxy salicylic acid and 2-diethylaminoethyl chloride. 5-Ethoxy salicylic acid was prepared by the reaction of diethylsulfate and gentisic acid in an alkaline medium followed by saponification and neutralization. 2-Diethylaminoethyl chloride was prepared as previously described (3). A crude yield of 59.4% of 2-diethylaminoethyl-5-ethoxy salicylate hydrochloride was obtained. Recrystallization from hot isobutanol gave white crystalline needles, m. p. 172.5–174.5°.

B.—3-(1-Methylpiperidyl)-methyl gentisate hydrochloride, 2-dimethylaminoethyl gentisate hydrochloride and 2-*n*-butylaminoethyl gentisate hydrochloride were prepared according to the precedure described by Feldkamp for acids (4). Three products were obtained as follows:

3-(1-Methylpiperidyl)-methyl Gentisate Hydrochloride.—One molar equivalent of the amino alcohol was reacted with one molar equivalent of gentisic acid as described. After refluxing for sixty-seven hours, 2.2 cc. of water had collected in the trap. The benzene was decanted and the residue extracted with three portions of acetone. The gray, crystalline, acetone-insoluble residue was set aside. The combined acetone portions were evaporated to a thick oil and the oil recyclized through the refluxing procedure for forty-four additional hours. The product was isolated as previously described. The combined gray crystalline solids corresponded to a yield of 83.6%. The product was recrystallized from ethanol and acetone (2:3) to give a very light tan, nearly white crystalline solid, m. p. 229–231°.

2-Dimethylaminoethyl Gentisate Hydrochloride.—One molar equivalent of the amino alcohol was reacted with one molar equivalent of gentisic acid as described. After refluxing for fifty hours, 1.8 cc. of water had collected in the trap. The product was isolated as described above. A yield of 34.8% of acetone-insoluble, gray crystals was obtained. Recyclization of the acetone-soluble portion did not yield any additional product. Recrystallization

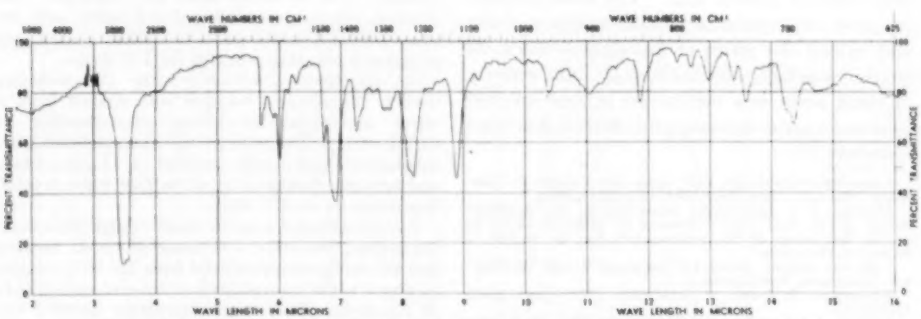


Fig. 2.—Infrared absorption spectrograph of 5-diphenylacetyl gentisic acid in Nujol®.

TABLE I.—ABSORPTION BANDS FOUND IN THE ESTERS OF GENTISIC ACID WITH OTHER CARBOXYLIC ACIDS SUSPENDED IN NUJOL®

Compound	Figure	Functional Group	Wave Length in Microns
A	Not given	Phenolic hydroxyl	2.90
		Aromatic C—H	3.2
		Ester carbonyl	5.75
		Acid carbonyl	5.93
		Phenyl ring	6.15 and 6.72
B	1	Ester carbonyl	5.70
		Acid carbonyl	5.95
		Phenyl ring	6.15 and 6.70
C	2	Ester carbonyl	5.70
		Acid carbonyl	6.00
		Phenyl ring	6.15 and 6.75

A—5,5'-Digentisyl phthalate.
B—5-Diethylacetyl gentisic acid.
C—5-Diphenylacetyl gentisic acid.

TABLE II.—ABSORPTION BANDS FOUND IN THE ESTERS OF GENTISIC ACID WITH AMINO ALCOHOLS SUSPENDED IN NUJOL®

Compound	Figure	Functional Group	Wave Length in Microns
A	Not given	Amine nitrogen	3.95
		Hydrogen chloride	4.10
		Acid carbonyl	5.95
		Phenyl ring	6.15 and 6.7
		Ether	9.17
B	3	Aromatic C—H	3.17
		Hydrogen chloride	3.8 and 4.2
		Acid carbonyl	6.0
		Phenyl ring	6.7
C	4	Aromatic C—H	3.15
		Hydrogen chloride	3.8 and 4.1
		Acid carbonyl	5.95
		Phenyl ring	6.7
D	Not given	Aromatic C—H	3.15
		Hydrogen chloride	3.8 and 4.1
		Acid carbonyl	5.93
		Phenyl ring	6.8

A—2-Diethylaminoethyl-5-ethoxy salicylate hydrochloride.
B—2-*n*-Butylaminoethyl gentisate hydrochloride.
C—2-Dimethylaminoethyl gentisate hydrochloride.
D—3-(1-Methyl piperidyl)-methyl gentisate hydrochloride.

from hot ethanol and a second recrystallization from ethanol, acetone, and benzene (1:1:1) gave a gray, crystalline solid, m. p. 198–199°.

2-n-Butylaminoethyl Gentisate Hydrochloride.—Two molar equivalents of the amino alcohol were reacted with one molar equivalent of gentisic acid as described. After refluxing thirty hours, water

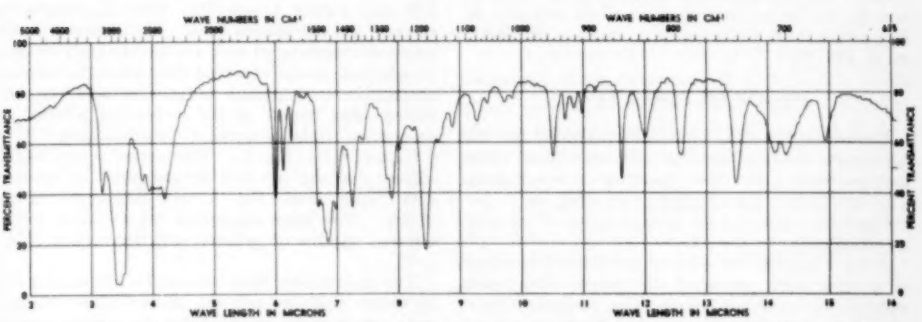


Fig. 3.—Infrared absorption spectrograph of 2-*n*-butylaminoethyl gentisate hydrochloride in Nujol®.

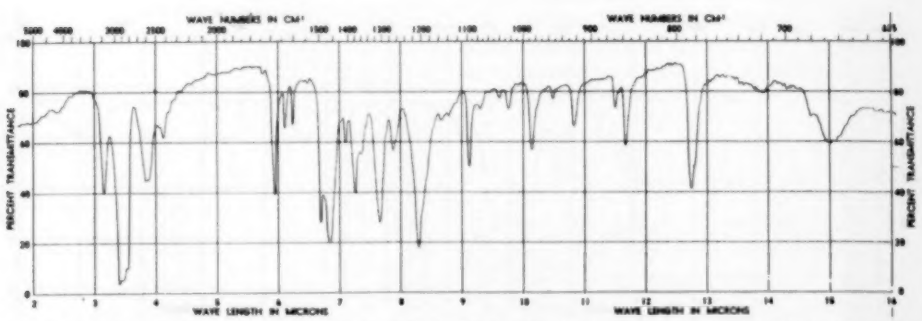
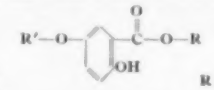


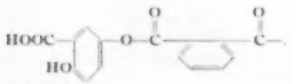
Fig. 4.—Infrared absorption spectrograph of 2-dimethylaminoethyl gentisate hydrochloride in Nujol®.

TABLE III.—ANALYTICAL DATA FOR THE RECENTLY PREPARED DERIVATIVES OF GENTISIC ACID



R′	R	Calculated N. E.[a]	Experimental N. E.	Carbon Content—Calcd.	Carbon Content—Found	Hydrogen Content—Calcd.	Hydrogen Content—Found
$C_{13}H_9O_6$—[b]	—H	219	224	60.28	60.18	3.22	3.32
$(C_6H_5)_2CHCO$—	—H	348	347	72.41	72.66	4.63	4.82
$(C_2H_5)_2CHCO$—	—H	252	254	61.89	62.15	6.39	6.40
C_2H_5—	$—C_2H_4N(C_2H_5)_2 \cdot HCl$	318	319	56.54	56.77	7.60	7.60
H—	—CH_2— (S) —N—$CH_2 \cdot HCl$	302	301	55.72	55.82	6.68	6.52
H—	$—C_2H_4N(CH_3)_2 \cdot HCl$	262	269	1.48	50.55	6.16	6.43
H—	$—C_2H_4NHC_4H_9 \cdot HCl$	290	[c]	53.88	54.02	6.96	8.10[c]

[a] Neutralization equivalent.
[b] $C_{13}H_9O_6$—represents



[c] See discussion.

ceased to be evolved from the reaction, and only 0.8 cc. of water had collected in the trap. The product was isolated as described above. A yield of 31.5% of acetone-insoluble, white crystals were obtained. Recyclization of the acetone-soluble portion gave a negligible additional amount of product. The white crystals were washed with ether and anhydrous acetone and repeatedly recrystallized from ethanol-acetone and from ethanol-ethylacetate mixtures to give a light fluffy white needle crystalline compound, m. p. 198–202°.

PROOF OF STRUCTURE

Experimental.—The structures proposed for the seven new esters of gentisic acid were based upon four qualitative and three quantitative sets of data. The qualitative data include two color tests, one precipitation test and the interpretation of infrared spectrographs.

Ferric Chloride Test.—All of the seven new esters of gentisic acid produced the purple color with aqueous ferric chloride solution that is characteristic of orthohydroxy aromatic acids. Nash, *et al.*, reported identical results with similar esters (1).

Beilstein Test.—The hydrochloride salts of the gentisic acid esters with amino alcohols gave a positive Beilstein test.

Silver Nitrate Test.—The hydrochloride salts of the gentisic acid esters with amino alcohols gave a white precipitate with aqueous silver nitrate solution.

Infrared Spectrographs.—Utilizing the same source of reference data as Nash, *et al.* (5), it was found that the infrared absorption spectrographs of these new esters, when each was suspended in Nujol®, contained the expected absorption maxima due to the presence of the expected functional groups (Figs. 1–4 and Tables I and II). Only representative spectra are reproduced here. The spectra of the esters not reproduced here are qualitatively similar. In addition, it was observed that when the infrared absorption spectrograph of gentisic acid was obtained using Nujol® as the suspending solvent, an absorption triplet occurred at approximately 5.95 μ, 6.1 μ, and 6.3 μ (Fig. 5). When ether is used as the solvent for the infrared spectrograph of gentisic acid (Nash, *et al.*, Fig. 1, [5]), this triplet is not visible. This same absorption triplet occurs in the infrared spectra of gentisic acid derivatives (Figs. 1–4).

The quantitative data consisted of the carbon and hydrogen analyses and the neutralization equivalents which were obtained by the potentiometric titration of a hydroalcoholic solution of the ester

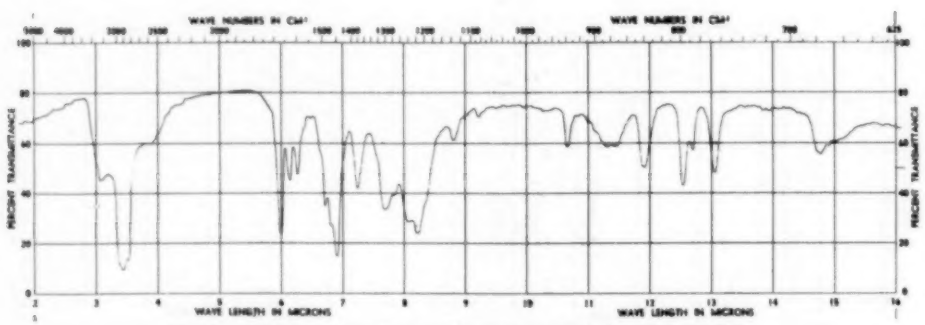


Fig. 5.—Infrared absorption spectrograph of gentisic acid in Nujol®.

TABLE IV.—SOME PHYSICAL PROPERTIES OF THE RECENTLY PREPARED DERIVATIVES OF GENTISIC ACID

R′	R	M. P., °C.	Ferric Chloride Test	Silver Nitrate Test	Beilstein Test	Solubilities[a] W	Al	E.	Ac.	Me.	P. E.	Is. B.
$C_{11}H_9O_5$[b]	—H	252–253	+	[c]	:	I[d]	S[e]	S, S.[f]	S	:	I	:
$(C_2H_5)_2CHCO$—	—H	200.5–201.5	++	:	:	I	S	I	S	:	I	S
$(C_2H_5)_2CHCO$—	—H	111.2–113.2	++	:	:	I	S	S	S	:	I	:
C_2H_5—	$—C_2H_4N(C_2H_5)_2·HCl$	172.5–174.5	+	+	+	S	S	I	I	S	I	S
H—	$—CH_2$ ·HCl	229–231	+	+	+	S	S	I	I	S	I	S S
H—	$—C_2H_4N(CH_3)_2·HCl$	198–199	++	++	++	S	S	I	I	S	I	S
H—	$—C_2H_4NHC_4H_9·HCl$	198–202	++	++	++	S	S	I	I	S	I	S

[a] W.—water, Al—ethanol, E.—diethyl ether, Ac.—acetone, Me.—methanol, P.E.—petroleum ether, Is. B.—isobutanol.
[b] $C_{11}H_9O_5$. For structure see Table III.
[c] Value not determined.
[d] Insoluble.
[e] Soluble.
[f] Slightly soluble.

with approximately 0.01 *N* sodium hydroxide solutions. The results of the quantitative tests are given in Table III.

DISCUSSION

The qualitative and quantitative data establish the structures of these new esters. The esters of gentisic acid with amino alcohols contain a carbonyl group, hydrogen chloride, a phenyl ring, and are monobasic. The esters of gentisic acid with other carboxylic acids contained two carbonyl groups, one free as a carboxyl group and one in an ester group; the diester was dibasic; the monoesters were monobasic.

The constitution of the compound believed to be 2-*n*-butylaminoethyl gentisate hydrochloride is somewhat in doubt because of high hydrogen content and inconstant neutralization equivalent values. These discrepancies are believed to be due to different degrees or kinds of solvation in which either acetone, alcohol, or water may be involved. The infrared spectrograph assures that the compound is a gentisate derivative.

SUMMARY AND CONCLUSIONS

The physical properties and the results of three of the qualitative tests of the recently prepared derivatives of gentisic acid are summarized in Table IV.

1. Seven new derivatives of gentisic acid were prepared: three were esters of gentisic acid with other carboxylic acids, three were esters of gentisic acid with amino alcohols, and one was an ester of a substituted gentisic acid with an amino alcohol.

2. All of the compounds were analyzed for carbon and hydrogen content and the molecular weight confirmed by the neutralization equivalent.

3. The proposed structures of the seven new derivatives of gentisic acid were confirmed by a positive ferric chloride test and the interpretation of their infrared absorption spectrographs.

4. Gentisic acid and its derivatives possess a characteristic infrared absorption triplet absorbing at approximately 5.9 μ, 6.1 μ, and 6.3 μ, when suspended in Nujol®.

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Some New Derivatives of Gentisic Acid*

II. Pharmacological Tests Upon Some New Derivatives of Gentisic Acid

By W. E. MOORE,† F. W. BOPE,‡ and B. V. CHRISTENSEN§

Seven new esters of gentisic acid were subjected to four selected pharmacological testing procedures: acute toxicity, antispasmodic activity, local anesthetic activity, and anticonvulsant activity. All of the seven new esters were more toxic than the parent, gentisic acid, when given intraperitoneally in mice in a soluble form. Of the four esters with amino alcohols, only 2-diethylaminoethyl-5-ethoxy salicylate hydrochloride possessed antispasmodic or local anesthetic activity. None of the four esters of gentisic acid tested for anticonvulsant activity possessed this activity.

THE ACUTE TOXICITIES of seven new esters of gentisic acid were determined by intraperitoneal injection into white mice. The hydrochlorides of the esters of gentisic acid with amino alcohols were water-soluble and were given as such. The esters of gentisic acid with other carboxylic acids were given as their water-soluble sodium salts.

The esters of gentisic acid with amino alcohols were screened for antispasmodic and local anesthetic activity due to their similarity in chemical structure to compounds now in use for these actions.

The esters of gentisic acid with other carboxylic acids and the gentisic acid ester, believed to be 2-*n*-butyl-aminoethyl gentisate hydrochloride, were screened for anticonvulsant activity because these compounds, when tested for acute toxicity, did not cause convulsions in mice preceding death.

EXPERIMENTAL

Acute Toxicity.—The procedure followed and the method of evaluation employed were essentially those previously described by Nash, *et al.* (1). The only modification was that the route of administration was by intraperitoneal injection of a soluble salt.

The results are shown in Tables I and II. These integrated data are illustrated graphically in Figs. 1 and 2.

The pattern of toxicities of these new esters of gentisic acid follows that found by Nash *et al.* (1). The amino alcohol esters were more toxic than the esters with other carboxylic acids and both were more toxic than the parent, gentisic acid. This relationship shows that the difference in toxicity is not due to solubility, as suggested by Nash, *et al.* (1), but is rather a function of structure.

There was a distinct difference in the manner in which death was produced between the two types of esters. All of the esters with amino alcohols in which the amino group was tertiary caused severe clonic convulsions before death. The compound supposed to be an ester in which the amino group was secondary, as well as the esters with other carboxylic acids did not cause convulsions before death. Nash, *et al.* (1), had found that by intravenous injection both types of esters caused convulsions before death.

Antispasmodic Activity.—The procedure followed was that of the standard method of testing drugs

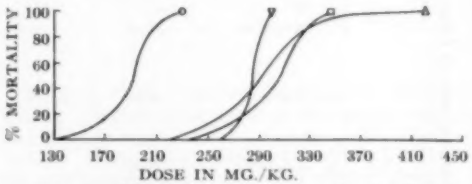

Fig. 1.—Mortality curves—I. ○—2-*n*-Butyl-aminoethyl gentisate HCl. ▽—3-(1-Methylpiperidyl)-methyl gentisate HCl. □—2-Dimethylaminoethyl gentisate HCl. Δ—2-Diethylaminoethyl-5-ethoxy salicylate HCl.

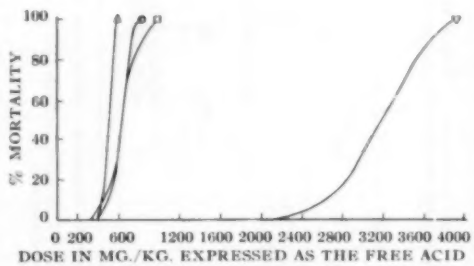

Fig. 2.—Mortality curves—II. Δ—5-Diphenylacetyl gentisic acid. ○—5-Diethylacetyl gentisic acid. □—5,5′-Digentisyl phthalate. ▽—Gentisic acid.

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Abstract of a dissertation submitted to the Graduate School of The Ohio State University by Willis E. Moore in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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TABLE I.—TOXICITY OF ESTERS OF GENTISIC ACID WITH AMINO ALCOHOLS

Compound	No. of Animals	Experimental Data: Dose, mg./Kg.	Alive	Dead	Integrated Data: Alive	Dead	Total	Mortality, %	LD50, mg./Kg.
A	12	218	12	0	26	0	26	0	
	12	257	9	3	14	3	17	18	
	12	307	4	8	5	11	16	69	
	12	345	1	11	1	22	23	96	
	12	420	0	12	0	34	34	100	289
B	12	233	12	0	33	0	33	0	
	12	257	9	3	21	3	24	13	
	12	300	9	3	12	6	18	33	
	12	321	3	9	3	15	18	84	
	12	346	0	12	0	27	27	100	305
C	12	260	12	0	36	0	36	0	
	12	270	10	2	24	2	26	8	
	12	280	9	3	14	5	19	26	
	12	290	5	7	5	12	17	71	
	12	300	0	12	0	24	24	100	285
D	12	130	12	0	43	0	43	0	
	12	150	11	1	32	1	33	3	
	12	170	8	4	20	5	25	20	
	12	190	9	3	12	8	20	40	
	12	210	3	9	3	17	20	85	
	12	230	0	12	0	31	31	100	193

A = 2-Diethylaminoethyl-5-ethoxy salicylate hydrochloride.
B = 2-Dimethylaminoethyl gentisate hydrochloride.
C = 3-(1-Methylpiperidyl)-methyl gentisate hydrochloride.
D = 2-*n*-Butylaminoethyl gentisate hydrochloride.

upon an isolated muscle strip (2). The muscle used was the isolated intestinal strip of a rabbit which had been starved for forty-eight hours. The four compounds tested were dissolved in sufficient distilled water so that when 2 ml. of test solution was diluted with 98 ml. of bath solution, the concentration was equal to 1 part per 100,000 parts. Papaverine hydrochloride was chosen as the standard and designated as *X*.

2-Diethylaminoethyl-5-ethoxy salicylate hydrochloride was designated as *A;* 3-(1-methylpiperidyl)-methyl gentisate hydrochloride was designated as *B;* 2-dimethylaminoethyl gentisate hydrochloride was designated as *C;* and 2-*n*-butylaminoethyl gentisate hydrochloride was designated as *D*.

A, *B*, *C*, and *D* were tested in comparison to *X* during four and one-half hours. The intestinal strip was still responding normally to *X* at the end of the period.

The results are shown in Table IV. Figure 3 illustrates the comparative actions of *A* to *X*.

Of the four esters of gentisic acid with amino alcohols, only 2-diethylaminoethyl-5-ethoxy salicylate hydrochloride possessed any appreciable

TABLE II.—TOXICITY OF ESTERS OF GENTISIC ACID WITH OTHER CARBOXYLIC ACIDS AND OF GENTISIC ACID AS THEIR SODIUM SALTS

Compound	No. of Animals	Experimental Data: Dose, mg./Kg.	Alive	Dead	Integrated Data: Alive	Dead	Total	Mortality, %	LD50, mg./Kg. of the Free Acid
E	12	400	12	0	34	0	34	0	
	12	450	9	3	22	3	25	12	
	12	500	8	4	13	7	20	35	
	12	550	5	7	5	14	19	74	
	12	600	0	12	0	26	26	100	520
F	12	350	12	0	41	0	41	0	
	12	450	10	2	29	2	31	6	
	12	550	8	4	19	6	25	24	
	12	650	10	2	11	8	19	42	
	12	750	1	11	1	19	20	95	
	12	850	0	12	0	31	31	100	650
G	12	400	12	0	37	0	37	0	
	12	500	10	2	25	2	27	7	
	12	600	7	5	15	7	22	32	
	12	700	5	7	8	14	22	64	
	12	800	3	9	3	23	27	85	
	12	1,000	0	12	0	35	35	100	650
H	12	2,000	12	0	36	0	36	0	
	12	2,500	11	1	24	1	25	4	
	12	3,000	8	4	13	5	18	28	
	12	3,500	5	7	5	12	17	71	
	12	4,000	0	12	0	24	24	100	3,250

E = 5-Diphenylacetyl gentisic acid.
F = 5-Diethylacetyl gentisic acid.
G = 5,5'-Digentisyl phthalate.
H = Gentisic acid.

TABLE III.—LOCAL ANESTHETIC EFFECT OF THE ESTERS OF GENTISIC ACID WITH AMINO ALCOHOLS COMPARED TO PROCAINE HYDROCHLORIDE

Compound	Time for Anesthesia, Minutes	Standard Deviation, Minutes	Fiducial Limits, 0.95 Probability, Minutes	Reversibility
A	7.1	1.8	3.2–11.0	Reversible
B	9.9	1.7	6.1–13.7	Reversible
C	26.3	6.4	12.2–40.4	Reversible
D	18.5	3.1	11.7–25.3	Not reversible
E	23.2	6.2	9.5–36.9	Reversible

A = Procaine hydrochloride.
B = 2-Diethylaminoethyl-5-ethoxy salicylate hydrochloride.
C = 3-(1-Methyl piperidyl)-methyl gentisate hydrochloride.
D = 2-Dimethylaminoethyl gentisate hydrochloride.
E = 2-*n*-Butylaminoethyl gentisate hydrochloride.

antispasmodic activity. The other three esters possessed little or no antispasmodic activity in concentrations of even 1:50,000.

Local Anesthetic Activity.—Local anesthetic action was tested upon the isolated sciatic nerve of a frog using the Ene Volt® inductorium manufactured by Gorrell and Gorrell as the type of stimulation. The isolated sciatic nerve itself was stimulated anterior to the site of application of the drug. Summer frogs stored at 5° in a shallow tray of water containing 0.15% sodium chloride and a trace amount of potassium permanganate were used. Before testing, the frogs were given a decerebration pithing and allowed to recover during fifteen minutes. The frog was then pinned to a frog board and the sciatic nerve was isolated from the thigh. The sciatic nerve was then placed in a paraffin block which contained a well for the introduction of the solution to be tested.

Preliminary testing indicated that the isolation of the nerve and its insertion into the paraffin block caused sufficient trauma that response to sensory stimuli was erratic or absent. The minimum threshold to electrical shock to cause flexing of the leg was determined for each leg. In those cases in which the threshold stimulus was less than 0.05 volt, a stimulus of 0.05 volt was used. In those cases in which the threshold stimulus was more than 0.05 volt, the threshold stimulus was increased by 0.1 volt.

A 1% solution of procaine hydrochloride was chosen as the standard local anesthetic. The compounds to be tested were used in the same concentration.

Fig. 3.—Antispasmodic activity of 2-diethylaminoethyl-5-ethoxy salicylate hydrochloride. *A* = 1:100,000 concentration of 2-diethylaminoethyl-5-ethoxy salicylate hydrochloride. *Y* = tissue washed twice with bath solution. *X* = 1:100,000 concentration of papaverine hydrochloride.

After the proper voltage for motor stimulation had been chosen, five drops of the test solution were placed into the paraffin well, through which passed the sciatic nerve. The end point was chosen to be the lack of response to the determined electrical stimulus when the stimulus was applied to the spinal side of the block. The results are reported in terms of the time in minutes required for each compound tested to produce blocking of motor response to the determined electrical stimulus. Both legs of the same frog were used and the data are the result of the testing of 12 legs or 6 frogs.

After anesthesia had been induced, the compound was removed and the ability of the sciatic nerve to recover was determined. If recovery did not occur within one and one-half hours, the induced anesthesia was considered to be irreversible.

TABLE IV.—PHARMACOLOGICAL EFFECTS OF SOME NEW ESTERS OF GENTISIC ACID IN FOUR SELECTED PHARMACOLOGICAL SCREENING PROCEDURES

Compound	LD$_{50}$, mg./Kg.	Antispasmodic Effect[b]	Local Anesthetic Effect[c]	Anticonvulsant Effect[d]
A[a]	650	Not tested	Not tested	None
B[a]	520	Not tested	Not tested	None
C[a]	650	Not tested	Not tested	None
D	289	Some	Some	Not tested
E	285	Slight	Very slight	Not tested
F	305	None	Very slight	Not tested
G	193	None	Very slight	None

A = 5,5′-Digentisyl phthalate.
B = 5-Diphenylacetyl gentisic acid.
C = 5-Diethylacetyl gentisic acid.
D = 2-Diethylaminoethyl-5-ethoxy salicylate hydrochloride.
E = 3-(1-Methyl piperidyl)-methyl gentisate hydrochloride.
F = 2-Dimethylaminoethyl gentisate hydrochloride.
G = 2-*n*-Butylaminoethyl gentisate hydrochloride.
[a] As the sodium salt.
[b] Isolated rabbit intestine, compared to papaverine hydrochloride.
[c] Isolated frog sciatic nerve, compared to procaine hydrochloride.
[d] Protection of mice against Metrazol® induced death.

The results, which have been evaluated by the method of the sum of the squares (3), are presented in Table III.

The most active of the esters of gentisic acid with amino alcohols was the compound most closely related to procaine hydrochloride. The effect of 2-diethylaminoethyl-5-ethoxy salicylate hydrochloride (*B*) was, however, not as great as an equal concentration of procaine hydrochloride (*A*). The other three esters possessed a very slight anesthetic potency that was somewhat inconsistent as shown by the spread of the fiducial limits of *C*, *D*, and *E* (Table III). 2-Dimethylaminoethyl gentisate hydrochloride (*D*) was apparently neurotoxic, for the nerve did not recover normal response within one and one-half hours.

Anticonvulsant Activity.—White Swiss mice obtained from the same source of supply, weighing 15–25 Gm. were used as the test animals. They were housed in standard cages at the prevailing conditions of humidity and temperature and were observed for twenty-four hours prior to use. They were fed on unlimited amounts of Purina Dog Checkers and water. Before testing, the mice were starved for twenty-four hours to standardize body weight. Their water supply was unlimited. The esters tested were 5,5′-digentisylphthalate, 5-diphenylacetyl gentisic acid, 5-diethylacetyl gentisic acid, and 2-*n*-butylaminoethyl gentisate hydrochloride. The esters of gentisic acid with other carboxylic acids were solubilized as their sodium salts. 2-*n*-Butylaminoethyl gentisate was soluble as the hydrochloride salt. The dosages used were the largest doses producing no deaths in the series as determined in the section on acute toxicity. Metrazol®, whose LD_{99} has been reported to be 120 mg./Kg. (4) was chosen as the convulsant. The dose of each of the four esters of gentisic acid was given by intraperitoneal injection to 12 mice. At the end of ten minutes and twenty minutes, 6 mice received the LD_{99} of Metrazol® by subcutaneous injection. Therefore, 12 mice received the dose of the ester and the LD_{99} dose of Metrazol®.

The doses used of the selected esters of gentisic acid exerted no protective action against death induced by Metrazol®. All the mice died following the typical convulsion which was induced by Metrazol®.

SUMMARY AND CONCLUSIONS

The pharmacological effects of the new derivatives of gentisic acid in four selected pharmacological screening procedures are presented in Table IV.

1. The most toxic of the esters of gentisic acid with other carboxylic acids was 5-diphenylacetyl gentisic acid with an LD_{50} of 520 mg./Kg., expressed as the free acid. The least toxic of these esters were 5-diethylacetyl gentisic acid and 5,5′-digentisyl phthalate with an LD_{50} of 650 mg./Kg. each, expressed as the free acid.

2. The most toxic of the esters of gentisic acid with amino alcohols was the supposed 2-*n*-butylaminoethyl gentisate hydrochloride with an LD_{50} of 193 mg./Kg. The least toxic of these esters was 2-dimethylaminoethyl gentisate hydrochloride with an LD_{50} of 305 mg./Kg.

3. 2-Diethylaminoethyl-5-ethoxy salicylate hydrochloride is somewhat less active than papaverine hydrochloride as an antispasmodic agent.

4. 2-Diethylaminoethyl-5-ethoxy salicylate hydrochloride produced motor anesthesia only slightly slower than procaine hydrochloride.

5. Neither the three esters of gentisic acid with other carboxylic acids nor 2-*n*-butylaminoethyl gentisate hydrochloride possessed sufficient anticonvulsant activity to protect mice from death produced by a lethal dose of Metrazol®.

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WHO MAKES IT?

The Registry of Rare Chemicals, Armour Research Foundation of the Illinois Institute of Technology, 35 West 33rd Street, Chicago 16, Ill., seeks information on sources of supply of the following chemicals:

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Sodium lauroyl sarcoside
2,5-Dimethyl-1,4-dioxane
1-Nitroheptane
3-Methyl-2-naphthoic acid
Sinigrin
Gentisic aldehyde
Mycophenolic acid
Lanosterol
Esculetin
Elemicin
Bromelin
Creatininease

Preparation of Some Steroids in Microcrystalline Form by Rapid Freeze-Sublimation Technique*

By A. P. LEMBERGER,† T. HIGUCHI, L. W. BUSSE, J. V. SWINTOSKY,‡ and D. E. WURSTER

Since it is sometimes desirable that insoluble therapeutic agents, such as steroids, in aqueous suspensions intended for parenteral use be present as extremely small particles, a procedure for acquiring powders in this state was investigated. A condensation method utilizing a rapid freezing-sublimation drying technique was found to be suitable for the preparation of cholesterol, pregnenolone, and Δ^4-androstene-3,17-dione in the form of submicron particles. The average particle size of the powders obtained by this procedure was calculated from data obtained by determining the specific surface area. Data are given to show that the average particle size obtained is influenced by the steroid chosen, the concentration of the steroid in the initial solution, the solvent used, the temperatures employed in the process, and the presence of certain additives.

IT IS SOMETIMES desirable to administer slightly soluble medicinal agents parenterally in the form of suspensions. When this route of administration is employed, the suspended particles are introduced into the tissues and remain imbedded while gradual solution by the tissue fluids takes place. The particle size of these suspended particles may play a vital role in determining the amount of irritation produced at the site of injection and definitely influences the rate at which the agent is absorbed (1).

Particles in a fine state of subdivision can be produced either by the comminution of larger particles or by the condensation of molecules or molecular aggregates. The ultimate size of the particles obtained when a condensation method is employed is dependent upon the rate of nucleation for new crystals and the rate of growth of preformed crystals. Previous investigations have shown that the rate of nuclei formation and crystal growth is influenced by the degree of supercooling of a melt (2, 3), supersaturation of a solution (3, 4, 5, 8, 11), the viscosity of the system (6, 7, 9), mechanical influences (6, 8), and the presence of foreign substances (2, 3, 7) or impurities (10, 13–20).

This study is concerned with a condensation method of producing certain steroids in a microcrystalline state of subdivision with possible application to the preparation of suspensions of steroids intended for parenteral administration. The investigation was carried out on the hypothesis that extremely fine powders of steroids can be prepared by forming solutions of the materials in an organic solvent, rapidly freezing the solutions, and removing the solvent by sublimation. It was hoped that this approach would result in massive nucleation of the supersaturated solution, but that only slight growth of the formed nuclei would occur. The experimental results obtained appear to bear this out.

EXPERIMENTAL

Procedure for Preparation of Frozen Solutions.—A chloroform or carbon tetrachloride solution of the steroid, with or without additives, was filtered and then allowed to drop from a separatory funnel directly into the refrigerant contained in a wide-mouth, pint Dewar flask at such a rate that discrete droplets of solution were formed before they came in contact with the freezing liquid. The freezing liquid employed was either liquid air or liquid nitrogen.

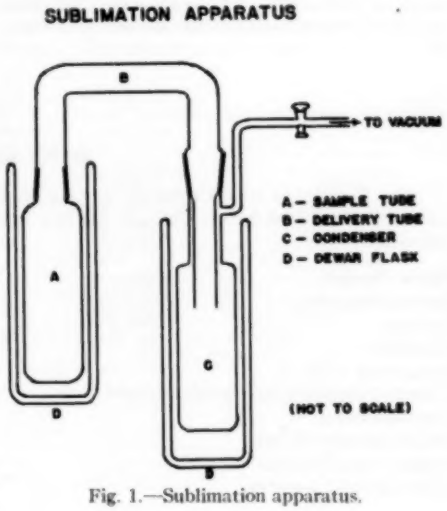

Fig. 1.—Sublimation apparatus.

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The paper is based on a dissertation submitted by August P. Lemberger to the Graduate School of the University of Wisconsin in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
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Procedure for Removal of Solvent.—The sublimation of the solvent from the frozen mass was accomplished in the freeze-drying apparatus shown in Fig. 1. A Cenco Pressovac vacuum pump together, in some instances, with an oil diffusion pump was used as the vacuum source. The frozen solution was transferred to the previously chilled sample tube with an excess of freezing liquid (liquid air or liquid nitrogen) and the sample tube placed on the drying apparatus. Both Dewar flasks were filled with liquid air and set in place. Then, under the influence of the vacuum, the excess freezing liquid was evaporated and the sample degassed. When the pressure of the system had been reduced to the point where a blue discharge from a Tesla coil was observed, the sublimation process was started by immersing the sample tube in a cooling medium considerably warmer than the coolant employed on the condenser. The rate of solvent removal was limited by the tendency of the subliming vapor to carry the light dry powder formed into the condenser. In the apparatus shown, approximately twenty-four hours were required to dry 50 ml. of the frozen solutions.

Procedure for Determination of Particle Size of the Powders.—The specific surface areas of the steroid powders obtained by the above procedure were determined by low temperature nitrogen adsorption (BET method) using the apparatus and procedure previously described by Swintosky, *et al.* (21–23).

RESULTS AND DISCUSSION

It is evident from the data presented in Tables I, II, and III that the over-all process is quite suited to the production of submicroscopic particles. Under optimum conditions the preparation of powders with surface average particle size of the order of $\frac{1}{4}$ micron (2,500 Å.) appears to be possible. In the following sections the influence of several variables of the process, including the steroid chosen, the concentration of the initial solution, the temperatures employed, the nature and amount of additives, on the fineness of the powders produced is discussed.

TABLE I.—PARTICLE SIZE OF VARIOUS CHOLESTEROL POWDERS OBTAINED BY THE RAPID-FREEZING METHOD

Samples Rapidly Frozen in Liquid Air, Maintained During Sublimation with an Acetone-Dry Ice Mixture			
Solvent for 10% Cholesterol Solutions	Specific[a] Surface Area ($M.^2/Gm.$)	Average Particle Diameter, Microns	Remarks
CCl_4	12.1	0.460	
CCl_4	12.0	0.463	
$CHCl_3$	21.6	0.258	
$CHCl_3$	21.9	0.254	
$CHCl_3$	13.2	0.422	0.1% Tween® mixture[b] added
$CHCl_3$	9.1	0.612	0.1% Tween® mixture[b] added
$CHCl_3$	17.5	0.318	0.1% Absolute ethanol added
$CHCl_3$	19.6	0.285	0.1% Absolute ethanol added
$CHCl_3$	3.2	1.768	10% Absolute methanol added
$CHCl_3$	3.0	1.876	10% Absolute methanol added

[a] Average of several determinations.
[b] Composition by weight. Tween® 20, 50%, Tween® 40, 40%, Tween® 81, 10%.

TABLE II.—PARTICLE SIZE OF PREGNENOLONE POWDER OBTAINED BY THE RAPID-FREEZING METHOD

Samples Rapidly Frozen in Liquid Air, Maintained During Sublimation with an Acetone-Dry Ice Mixture		
Concentration in Chloroform, %	Specific[a] Surface Area, $M.^2/Gm.$	Average Particle Diameter Microns
1	2.6	1.988
2.5	4.7	1.098
5	5.7	0.893
10	4.6	1.127
15	2.5	2.068

[a] Average of several determinations.

Nature of the Steroid.—It is to be expected that each compound would behave somewhat differently when subjected to the process since such factors as the relative solubility, ease of nucleation and crystal growth, etc., would influence the fineness of the resulting powder. In the present study it has been found, for example, that cholesterol yields powders as fine as $\frac{1}{4}$ micron whereas pregnenolone and Δ^4-androstene-3,17-dione produced powders with a particle size only a little less than one micron.

Effect of Concentration.—The concentration of steroid in the solution subjected to the rapid freezing process strongly influences the average particle size and possibly the nature of the final powder. This is evident in Figs. 2 and 3 where the specific surface area in square meters per gram of the resulting powder has been plotted against the initial concentration for cholesterol and pregnenolone. From these curves it is seen that in both instances a maximum specific surface area is reached. In the case of cholesterol the maximum occurs when the concentration in the frozen solution is approximately 10%, whereas, the maximum specific surface area of pregnenolone occurs when the concentration is approximately 5%.

That a critical concentration of steroid in the frozen solution exists at which the specific surface area of the powder obtained is maximal is seen from the plots. The lower specific surface areas observed when the steroid was present in concentrations less than the critical concentration are probably the result of crystal growth during the removal of the solvent. Later work in these laboratories has

TABLE III.—PARTICLE SIZE OF Δ^4-ANDROSTENE-3,17-DIONE POWDERS OBTAINED BY THE RAPID-FREEZING METHOD

Samples Rapidly Frozen in Liquid Air, Maintained During Sublimation with an Acetone-Dry Ice Mixture [a]		
Concentration in Carbon Tetrachloride, %	Specific[a] Surface Area, $M.^2/Gm.$	Average Particle Diameter, Microns
5	6.0	0.843
5	5.9	0.855

[a] Average of several determinations.

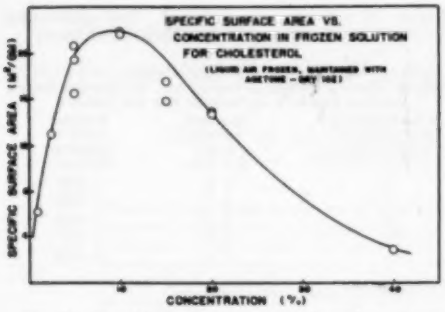

Fig. 2.—Specific surface area as a function of concentration in the frozen solution for cholesterol. Samples were frozen in liquid air and maintained with an acetone-dry ice bath during sublimation of the solvent.

shown that the steroid powders obtained in this process are sensitive to heat, the specific surface area decreasing under this influence.

Effect of Temperature.—Although the temperature of the refrigerant employed in the rapid freezing of the steroid solution probably influences the size of the final particles to some extent, this was not evident when the freezing medium was changed from liquid air to liquid nitrogen. The results of these runs are shown in Figs. 2 and 4.

The temperature of sublimation appears to have some effect on the particle size of the dry powders. When a cold air bath was substituted for the bath containing the acetone-dry ice mixture, the specific surface area decreased as can be seen by comparing Figs. 4 and 5. This indicates that growth of the particles occurs as the subliming sample approaches the melting temperature of the solvent. The effect

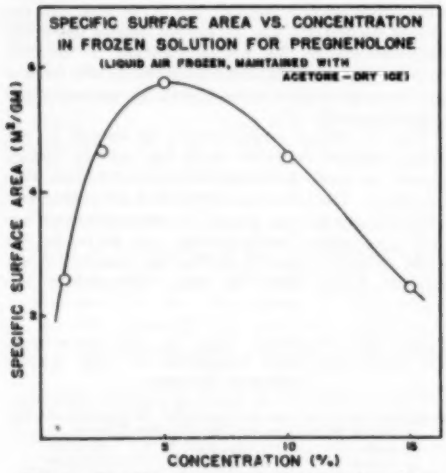
SPECIFIC SURFACE AREA VS. CONCENTRATION
IN FROZEN SOLUTION FOR PREGNENOLONE
(LIQUID AIR FROZEN, MAINTAINED WITH
ACETONE – DRY ICE)
SPECIFIC SURFACE AREA (M²/GM)
CONCENTRATION (%)

Fig. 3.—Specific surface area as a function of concentration in the frozen solution for pregnenolone. Samples were frozen in liquid air and maintained with an acetone-dry ice bath during sublimation of the solvent.

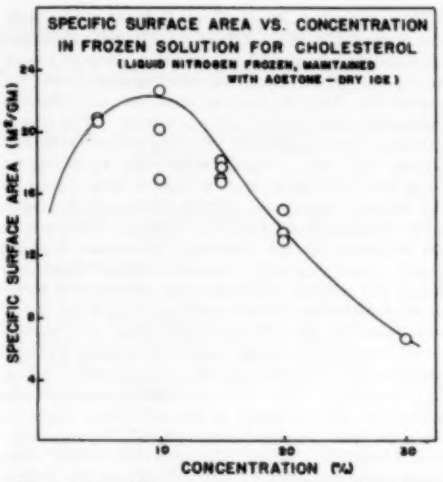
SPECIFIC SURFACE AREA VS. CONCENTRATION
IN FROZEN SOLUTION FOR CHOLESTEROL
(LIQUID NITROGEN FROZEN, MAINTAINED
WITH ACETONE – DRY ICE)
SPECIFIC SURFACE AREA (M²/GM)
CONCENTRATION (%)

Fig. 4.—Specific surface area as a function of concentration in the frozen solution for cholesterol. Samples were frozen in liquid nitrogen and maintained with an acetone-dry ice bath during sublimation of the solvent.

of temperature on the growth of the particles derived by this method will be shown in a subsequent report.

Effect of Solvent.—It is logical to assume that the solvent employed for the dissolution of the steroid in this procedure would have some effect on the particle size of the resulting powder. To investigate the effect of the solvent on the specific surface area of the resulting powder, carbon tetrachloride was used in place of chloroform. Cholesterol was used in this work and the results are shown in Table I. It is seen that a 10% solution of cholesterol in carbon tetrachloride gives rise to a powder with a specific surface area of about 12.1 $M.^2$ per gram while a similar solution in chloroform results in a powder with a specific surface area of about 21.8 $M.^2$ per gram.

Effect of Additives.—When absolute ethanol, absolute methanol, and the Tween® mixture alone were employed the specific surface areas of the prepared powders could be determined since the additive was either removed during sublimation or represented only a small total of the final product. The results of these procedures are shown in Table I.

It is seen that these agents reduced the specific surface area of the cholesterol powder. With absolute ethanol and absolute methanol the results may be explained by postulating a growth of the steroid particles in the alcoholic medium during the removal of the solvent. In the case of the Tween® mixture, the observed decrease may be due to the occluding of some of the pores in the steroid particles by the additive and the aggregation of the primary particles which were coated with a film of the Tween® mixture.

When caffeine and PVP (polyvinylpyrrolidone) were employed as additives in an effort to reduce the aggregation of particles during the sublimation procedure, the specific surface area of the resulting

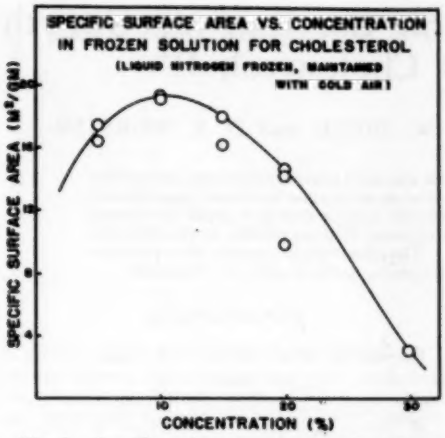

Fig. 5.—Specific surface area as a function of concentration in the frozen solution for cholesterol. Samples were frozen in liquid nitrogen and maintained with a cold air bath during sublimation of the solvent.

powders could not be used as an index to particle size because the additives represented a substantial portion of the final product. Analysis, therefore, consisted of preparing aqueous suspensions of the powders obtained and making visual observations.

With caffeine as the protective agent, the cholesterol in the resulting powder was readily wetted, but as the caffeine went into solution, the cholesterol particles immediately aggregated. When PVP was used as the protective agent, the resulting powder was also rapidly wetted and the tendency to aggregate was again noticed. Samples in which the Tween® mixture was used with PVP were very rapidly wetted and the resulting suspension showed no tendency toward coagulation of the cholesterol. On dilution of more concentrated suspensions produced with these powders, dispersions of almost colloidal characteristics resulted. These explorations were limited but from them it can be seen that many interesting possibilities exist for the application and improvement of this technique.

SUMMARY

1. Steroids can be prepared in the form of submicron particles by rapidly freezing solutions of them and removing the solvent by sublimation from the frozen mass.

2. The average particle size, calculated from the specific surface area, of powders produced by the rapid freezing-sublimation drying method is dependent upon the steroid employed, the concentration of steroid in the solution to be frozen, the temperature at which the frozen solution is maintained during the sublimation of the solvent, and the solvent used. The existence of a critical concentration for the steroid in the solution to be processed, at which the average particle size is minimal, has been demonstrated.

3. The addition of foreign substances to the steroid solution before freezing affects the average particle size of the powder obtained.

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Influence of Temperature on the Rate of Growth of Microcrystalline Cholesterol, II*

A. P. LEMBERGER,† T. HIGUCHI, L. W. BUSSE, and D. E. WURSTER

The influence of thermal aging at room and elevated temperatures on submicron cholesterol particles prepared by the freeze-drying technique has been investigated. It is shown that the rate of decrease in the specific surface area as a result of thermal aging is dependent upon the aging temperature and adheres closely to the relationship described by the Arrhenius equation. The theoretical aspects of a possible mechanism operative in the reduction of the specific surface area are discussed.

IT HAS BEEN SHOWN that certain steroids can be prepared in the form of extremely fine particles by rapidly freezing solutions of these steroids in organic solvents and removing the solvent from the frozen mass by sublimation (1). Because of the manner of their preparation it was felt that these powders may represent a metastable form of the steroid and that they may be subject to gradual changes on aging. In this report the results of an experimental investigation made of this effect are presented along with a theoretical discussion of their implications.

The process employed in the preparation of these powders was designed, in a sense, to leave the powders in as high an energetic state as possible. The steroids were dissolved in solvents to eliminate the intermolecular forces existent in the original substance and quick frozen, to favor either massive nucleation or to prevent nucleation almost completely. The solvents were then sublimed from the frozen mass. It is very doubtful that the solute existed in any well-defined crystalline state in either the frozen or the freshly dried state. Because of the highly energetic form of the fresh powders, however, the vapor pressures of the steroids in this form were probably abnormally high and the melting points abnormally low. This phenomenon occurring at room temperature can lead to the formation and growth of the stable configuration. Our experimental findings were in accord with this reasoning.

In the present investigation the effect of aging on an extremely fine powder form of cholesterol prepared by the freeze-drying technique has been studied. The physical changes in the powder were followed by determining the specific surface area by the nitrogen adsorption method.

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The paper is based on a dissertation submitted by August P. Lemberger to the Graduate School of the University of Wisconsin in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
† Fellow of the American Foundation for Pharmaceutical Education.

EXPERIMENTAL

Preparation of the Powder.—A large quantity of cholesterol was converted to the powder by the quick freeze-sublimation method previously described. The cholesterol solution employed was 10% w/v in chloroform.

Aging Procedure.—Fresh samples of the cholesterol powder were subjected separately to aging at room temperature and at 45°, 55°, and 64°. The runs made at elevated temperatures were carried out in a vacuum oven maintained at the several fixed temperatures by thermostatic control.

Determination of the Specific Surface Area.—The specific surface areas of parallel samples were determined by the Brunauer-Emmett-Teller nitrogen adsorption method as described previously by Swintosky, *et al.* (2, 3). Determinations were made on both samples initially and after each increment of the aging process. Additional samples were aged independently in some cases and their surface areas measured as a check on the reliability of the procedure.

RESULTS AND DISCUSSION

Experimental results show that storage at room temperature or at higher temperatures produces a definite decrease in the specific surface area of the freeze-dried cholesterol. This is evident in Figs. 1, 2, 3, and 4, showing the curves obtained when the

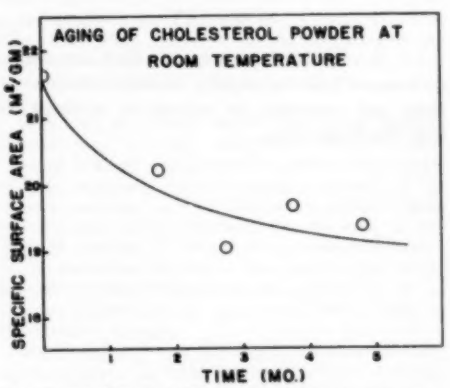

Fig. 1.—Aging of cholesterol powder at room temperature.

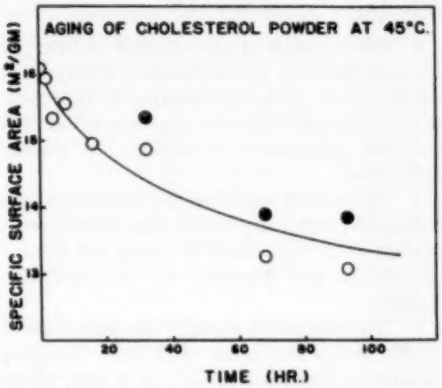

Fig. 2.—Aging of cholesterol powder at 45° C. ○—Average value for two parallel samples; ●—check determinations on independent samples.

specific surface area of the aged cholesterol sample is plotted as a function of the time of aging at room temperature, 45°, 55°, and 64°, respectively. The initial value for the room temperature run differed from the others because a different lot of powder was used. By comparing these curves it is evident that the decrease in specific surface area is affected by a process which is very sensitive to temperature. This indicates that the fundamental process responsible for the particle growth must have a high temperature coefficient.

It is possible to approximate the heat of activation of this phenomenon by application of the Arrhenius equation which states:

$$\log K = -\frac{\Delta H}{2.303R}\frac{1}{T} + \text{Constant}$$

where K is the rate constant of the process at absolute temperature, T, ΔH is the heat of activation, and R is the gas constant. Although it is impossible to calculate the absolute rate constant, a relative rate constant can be determined. This is based on the fact that for a given reaction taking place in a given system, the rate constant is inversely proportional to the time required for a fixed fraction of the reaction to take place. This is equally

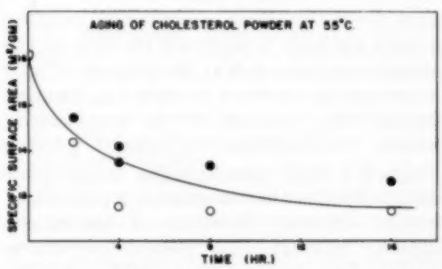

Fig. 3.—Aging of cholesterol powder at 55° C. ○—Average value for two parallel samples; ●—check determinations on independent samples.

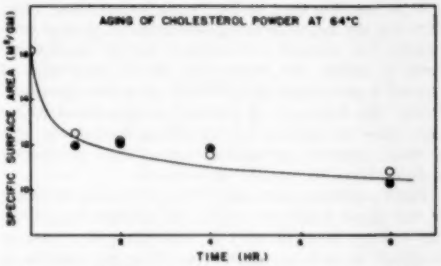

Fig. 4—Aging of cholesterol powder at 64° C. ○—Average value for two parallel samples; ●—check determination on independent samples.

true for zero order, first order, second order, etc., reactions.

Thus the slope of the line obtained by plotting log t', where t' is the time required for a fixed fraction of the reaction to take place, against $1/T$ is a measure of the heat of activation of the process. In Fig. 5 the logarithm of the time required for the specific surface area to decrease from the initial value of 16.1 to 14.0 M.²/Gm has been plotted against the reciprocal of the absolute temperature. The slope of this line multiplied by $2.303R$ corresponds to a heat of activation of approximately 60 kilocalories. It is acknowledged that this value may be substantially in error, but it is of the correct order of magnitude.

The high energy of activation for the process tends to rule out the possibility that the observed decrease in specific surface area is the result of a vaporization process wherein the molecules held with weak intermolecular bondings (unstable crystal positions) vaporize, only to condense on the larger particles in more stable orientations. Rather, the high energy requirement for the process would seem to point to a melting, a solid phase transition, or some other cooperative phenomenon concerning the active individual particles. Such cooperative phenomena can explain the very high temperature coefficient noted in the present case.

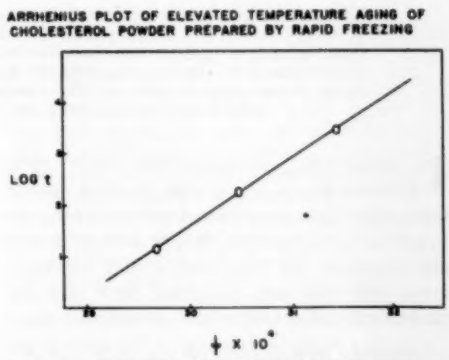

Fig. 5.—Plot of the logarithm of the time required for the specific surface area to decrease from 16.1 to 14.0 square meters per gram as a function of the reciprocal of the absolute temperature. The slope of this plot multiplied by $2.303R$ is equal to the heat of activation (approximately 60 kilo calories).

It is possible that in the formation of these powders by the rapid freezing-sublimation drying procedure the steroid precipitates in an amorphous form in which the orientation of the molecules of steroid leaves them in relatively unstable positions. Under the influence of thermal energy these molecules may be capable of reorienting themselves into a more orderly crystalline lattice thus producing a diminution in specific surface area.

Such a phenomenon may be looked upon as being of the phase transition type. In a phase transition the energy level of all the molecules of a system is raised to such an extent that they are capable of passing over one another freely, reaching a final stability either as a new crystal form or as a liquid. In this case, only those molecules in unstable positions would become capable of movement. These molecules could then seek a more stable position in the crystal lattice. The significance of this occurrence is realized by observing that a decrease in specific surface area from 16 to 12 $M.^2/Gm.$ corresponds to an increase in the bulk of the primary particle of more than 100%.

Although the results obtained in this work support the theoretical concepts given above, further experimentation is required before more definite statements can be made regarding the fundamental mechanism whereby the specific surface area is reduced.

SUMMARY

1. Investigation of the physical stability of powders prepared by sublimation drying of a rapidly frozen chloroform solution of cholesterol has revealed that this type of powder is unstable from the standpoint of retaining its specific surface area.

2. The rate at which the specific surface area decreases is highly dependent upon the temperature at which the powder is stored and follows the relationship described by the Arrhenius equation.

3. The mechanism whereby the diminution in specific surface area occurs is possibly through a cooperative phenomenon such as a solid phase transition, but further investigation is required for verification of such a postulation.

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The Physics of Tablet Compression

III. Design and Construction of an Instrumented Tableting Machine*

By T. HIGUCHI, EINO NELSON, and L. W. BUSSE

The design and performance of an instrumented tableting machine are presented. A Stokes Model A-3 single punch machine has been fitted with strain and displacement gauges to permit simultaneous recording of several variables including the force exerted by the upper punch, the force exerted on the lower punch, the movement of the upper punch, and the ejection force. Several fields of tablet research which can benefit by the use of this instrument are discussed.

IN PRECEDING communications from these laboratories (1, 2) several physical factors influencing the compressional process during the formation of compressed tablets were evaluated and discussed. In these studies, since the equipment employed only permitted force and displacement readings at rates of compression many times slower than those of commercial units, no attempt was made to determine the dynamic and energetic variables such as the influence of rates of compression, work of compression, frictional energies, etc., involved in the compressional process. To circumvent this difficulty, a Stokes Model A-3 single punch tablet machine[1] has been modified and instrumented to provide simultaneous electronic recordings of the several variables involved. The present communica-

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[1] Furnished through courtesy of F. J. Stokes Co., Philadelphia, Pa.

tion is concerned with the design, construction, and performance of this research instrument.

The machine which is described, together with its associated equipment, will simultaneously record any two of the following variables as functions of compression time: (*a*) the absolute force exerted by the upper punch; (*b*) the absolute force transmitted to the lower punch; and (*c*) the absolute displacement of the upper punch.

To find the relationship between any two of the above quantities it is only necessary to read the value of the variables from the record made at various time intervals in the compression process. In addition to the determination of the quantities above, the force necessary to remove the finished tablet from the die appears on the records transcribed by both the lower and upper punch forces.

REQUIREMENTS OF DESIGN

It was desired that the instrumented machine and its associated equipment be capable of measuring the compressional variables to the following degrees of accuracy and limits: (*a*) force exerted by the upper punch within 2% to 3,000 lbs., (*b*) force transmitted to the lower punch within 2% to 3,000 lbs., and (*c*) displacement of the upper punch to ±0.002 inch.

It was also desired that the response speed of the measuring devices and recording equipment be such that the recording lag time be less than one $^1/_{100}$ of a second.

These measurement requirements were chosen since they represent the approximate orders of magnitude and reproducibilities found in commercial processes. The total compressional force, for example, rarely exceeds 3,000 lbs. during industrial compression of tablets $^3/_8$ inch in diameter or smaller, the maximum design force in some smaller equipment being of this level. The one $^1/_{100}$ second response was taken since the actual compressional process in commercial rotaries occur within a few tenths of a second. Actually, this response speed is somewhat slower than would be desirable but was selected since it also represented the recording limitations of magnetic oscillographs.

INSTRUMENT DESIGN AND CONSTRUCTION

The requirements discussed above were easily met by use of strain gauges for force measurement and a differential transformer for displacement determinations. Since the outputs of these sensing devices are electrical signals, it was possible to amplify and record them by means of rapid response magnetic oscillographs.

Scheme of Instrumentation.—The modifications and additions made to the Stokes machine proper are shown on Fig. 1. The force exerted by the upper punch was determined by strain gauges bonded to the machine frame at *E* and *E'*. The movement of the upper punch was detected by the differential transformer marked *A* whose core moved with the upper punch by means of the linkage *D*. The force

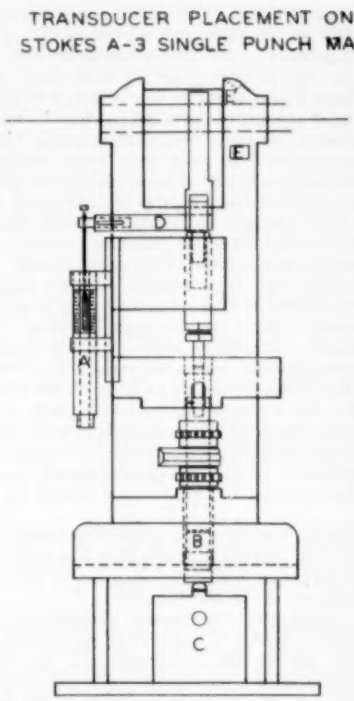

Fig. 1.—Schematic drawing of the instrumented Stokes Model A-3 tablet machine. *A*—Differential Transformer. *B*—Lower plunger extension. *C*—Load cell. *D*—Linkage to differential transformer core. *E* and *E'*—Strain gauges.

transmitted to the lower punch was determined with a Baldwin load cell which is labeled *C* on the drawing. The devices converted the mechanical effects to be measured into electrical signals which varied with the intensities of the mechanical effects under study. The signals from the transducers were fed to a Sanborn Twin Viso two-channel magnetic oscillograph equipped with strain gauge amplifiers.

Machine Drive.—The instrumented machine was modified to be driven by a $^1/_2$ horsepower electric motor through a hydraulic transmission (Vickers AA 16801 A), operating through a 25:1 worm gear speed reducer (Chicago Gear Works 1086). The hydraulic transmission permitted continuous variation of compression rate through a wide range of speeds and the worm gear speed reducer provided the necessary increase in torque on the tablet machines drive shaft to allow punching tablets at the maximum force without stalling. The drive motor, transmission, speed reducer, and tablet machine were all mounted on a heavy wooden table. The resulting assembly permitted dynamic studies at speeds varying from a punching rate of 7 tablets per minute to a rate about $^1/_{150}$ times as fast.

Measurement of Upper Punch Displacement.—The upper punch displacement was measured by a Schaevitz Model 500S-L linear variable differential transformer mounted on one side of the tablet machine's frame as shown in Fig. 1. This transformer consisted of three coaxial windings on a hol-

low ceramic cylinder. One winding was placed at the middle of the form and served as the transformer's primary. The two secondaries were adjacent to the primary, one on each side. The transformer had a movable core which was linked to the upper plunger of the tablet machine by means of a threaded rod and crossarm as shown in Fig. 1. The transformer was fixed to a brass mounting assembly which was bolted to one side of the tablet machine. The linkage of the transformer's core to the upper plunger and punch was such that as the machine went through a punching cycle the core executed the same motion as the upper punch.

The differential transformer's primary was excited by a 2,500 c. p. s. signal generated by an oscillator integral to Sanborn strain gauge amplifiers. The transformer's secondaries were connected in series opposition so that when the core was as much in the field of one secondary as it was in the field of the other, there was no net voltage output. Core movement away from this zero position resulted in a voltage developed across the connected secondaries. The magnitude of the voltage developed was directly proportional to the distance moved by the core.

The output voltage of the differential transformer was fed to the strain gauge amplifier and after suitable amplification and transformation it actuated the writing pen of the oscillograph.

The displacement-measuring assemblage was calibrated by means of a traveling microscope micrometer. A fine line scratched into the upper punch was used as a reference point. The distance traveled by the punch was measured by the micrometer and the sensitivity of the strain gauge amplifier was adjusted so that each chart division on the recorder paper was equal to the desired known punch displacement.

Measurement of Force on Lower Punch.—The force transmitted to the lower punch in the compression process was measured by using a Baldwin type "C" SR-4 load cell. In order to use this device, the tablet machine was mounted on a platform, there being sufficient room between the base of the machine and the platform to install the load cell. The placement of the load cell in relation to the machine and its lower plunger assembly is also shown in Fig. 1. The load cell was positioned so that its loading button was directly below the lower plunger assembly. An expandable pin, marked *C* on Fig. 1, was inserted between the loading button of the cell and the lower plunger assembly of the machine and extended to such length that the entire plunger assembly rested on the cell. By this arrangement the force which was exerted by the upper punch and transmitted through the tablet to the lower punch was sensed directly by the load cell.

The Baldwin load cell is a strain gauge device. It consists of a centrally positioned steel column in a cylindrical heavy steel case. Strain gauges are bonded to the column and when stress is applied, the resulting strain in the column is shared by the several gauges. The gauges are arranged in a Wheatstone bridge circuit, any strain on the column causing a change in the resistance of the gauges and consequently an unbalance in the bridge network. The degree of unbalance of the bridge network as manifested by a potential difference across its output is directly proportional to the force applied to the loading button of the load cell.

The Wheatstone bridge of the load cell was also excited by a 2,500 c. p. s. signal provided by the strain gauge amplifier used for lower punch force recording. The voltage output of the bridge resulting from application of force to the load cell was amplified by the strain gauge amplifier and actuated the writing pen of the oscillograph after suitable transformations in other sections of the amplifier. The load cell and associated equipment was calibrated by suspending a known weight on the cell's loading button by means of a 10:1 lever arm. The sensitivity of the strain gauge amplifier was variable over a wide range so that any desired range of force could be recorded.

Measurement of Force Exerted by Upper Punch.—The force exerted by the upper punch was determined by means of two strain gauges bonded to the frame of the machine as shown in Fig. 1. The strain gauge marked *E* on the figure was bonded to the underside of the yoke which supported the drive shaft on the drive to wheel side of the machine. The gage marked *E'* was bonded to the same yoke only on the side near its upper edge. Force exerted by the upper punch had its reaction on the drive shaft; the force on the drive shaft in turn tended to straighten the yoke which held it and consequently stretched gauge *E*. Gauge *E'*, being mounted near the top edge of the yoke and on the side, compressed in the same process. Gauges *E* and *E'* were arranged in opposite arms of a Wheatstone bridge network so that the response of the arrangement to measure force was due to the sum of the two resistance changes of the two gauges. This Wheatstone bridge circuit was also excited by a 2,500 c. p. s. signal from an oscillator. The output signal was handled in the same manner as was the signal resulting from lower punch force measurements. The strain gauges used were Baldwin SR-4 type A-13-2 having a resistance of 350 ohms each.

The gauging just described also allowed measurement of the force necessary to remove the tablet from the die after compression. If the tablet resisted being pushed free of the die by the lower punch, the resulting strain in the lift rod caused the yoke to tend to bend downward compressing gauge *E* on the underside of the yoke and stretching gauge *E'* on the side. The machine's lift rod is not shown on Fig. 1. On the machine it was parallel to the upper plunger assembly and was actuated by a cam fastened to the drive shaft of the machine.

TYPE AND NATURE OF DATA OBTAINABLE

Force and Displacement Measurements.—Typical oscillograph records of lower punch force and upper punch displacement are shown in Fig. 2. The lower half of the chart is the record of upper punch displacement and the upper half of the chart is the common record of the force transmitted to the lower punch, time being the parameter in both cases. That portion of each curve which is of interest in energy measurements consists of the parts which lie to the left of the maxima. These regions are transcribed when the tablet is made. This particular pair of curves was obtained by punching 570 mg. of a slugged sulfathiazole granulation sieved between 20 and 10 mesh screens. The original granulation was magnesium stearate lubricated.

A direct lower punch force *vs.* upper punch displacement curve is shown in Fig. 3. The data for

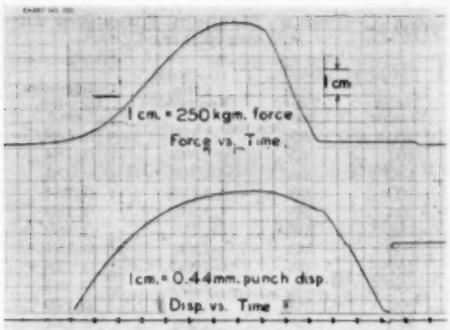

Fig. 2.—Reproduction of oscillographic record of lower punch force and upper punch displacement.

curves such as this were obtained from an oscillograph record like Fig. 2. This particular curve is a composite made from data obtained from punching two tablets at the speeds indicated on the diagram. The granulation and its weight were the same as that used to obtain the oscillograph record of Fig. 2. In the case of Fig. 3, the area under the curve represents mechanical work of 1.46 calories.

Before curves such as Fig. 3 can be plotted, it is necessary to correct the displacement data given on the oscillograph record for distortion of the machine parts under stress. The correction is determined by applying stress while the upper and lower punches are face to face and noting the displacement registered. The correction applied before Fig. 3 could be drawn was 0.02 mm. per 100 Kg. of force appearing on the lower punch.

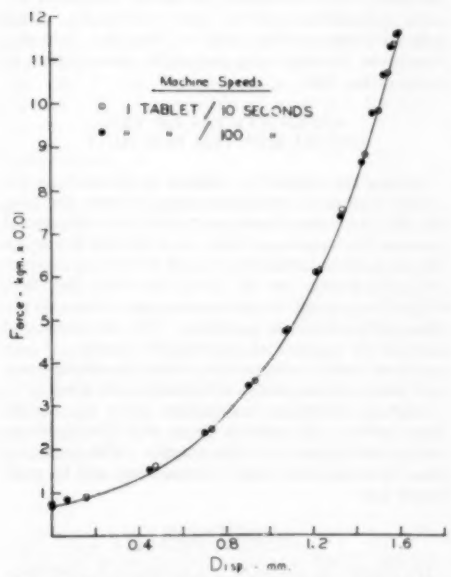

Fig. 3.—Lower punch force *vs.* upper punch displacement for two punching rates.

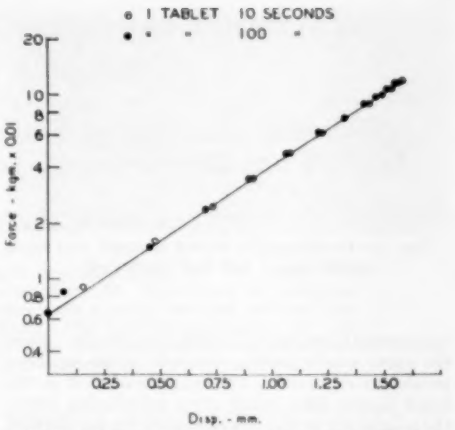

Fig. 4.—Lower punch force *vs.* upper punch displacement for two punching rates when force is plotted on logarithmic scale.

The data plotted in Fig. 3 on a linear scale are plotted in Fig. 4 with a logarithmic scale for force. The resulting straight line relationship is in agreement with previous findings (1, 2) obtained with slower set-ups.

Upper and Lower Punch Force Measurements.—Figure 5 shows the type of oscillograph record obtained when one channel of the oscillograph is used to measure upper punch force and the other to measure lower punch force. In this recording the upper curve is a record of the upper punch force and the lower curve of lower punch force. The gauging for determination of the upper punch force does not respond in a linear manner so it was necessary to correct this curve before the data were used.

The recording in Fig. 5 was made by punching 380 mg. of a sulfathiazole granulation containing 2.5% starch. No lubricant was added to the granulation. A $^3/_8$ inch flat-headed punch and die set was used in the machine.

Some very useful information can be obtained from curves of the type of Fig. 5. For example, the difference between the maximum value of force recorded by the upper and lower punches is a measure of the efficiency of a lubricant for overcoming wall friction. The portions of the two curves marked *A* and *B* give other useful information. That portion of the upper punch curve marked *A* is transcribed as the lower punch is pushing the tablet clear of the die. The recorder line drops below zero indicating that in this case 200 Kg. of force

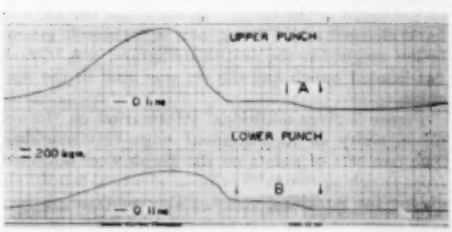

Fig. 5.—Oscillographic record of upper and lower punch force.

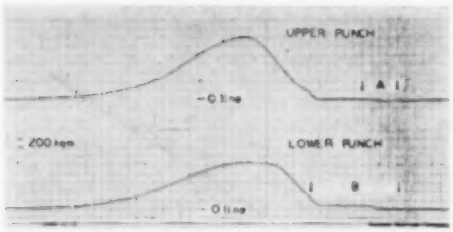

Fig. 6.—Oscillographic record of upper and lower punch force. Die wall lubricated.

was needed to remove the tablet from the die. How the upper punch gauging responds to this has been previously explained. The region marked *B* on the lower punch force curve gives information about the magnitude of the forces exerted by the finished tablet on the walls of the die. A comparison with the corresponding region on the upper punch force curve shows that the force being exerted by this punch is zero, yet 200 Kg. of force still register on the lower punch. This means that the tablet was exerting sufficient force against the walls of the die to prevent relaxation of the lower plunger assembly and load cell column beyond the indicated amount of force. Then, the minimum force which was required to remove this tablet from the die was 200 Kg. The apparent difference between the force read from the chart at *A* and *B* is due to the previously mentioned nonlinearity of response of the upper punch gauging.

The effect of lubricating the die wall on the force distribution just discussed can be seen from an examination of Fig. 6. In this case the die wall was thinly coated with white petrolatum but otherwise the tablet was made under the same conditions as the one giving the curves of Fig. 5. In the lubricated case only 70 Kg. of force was needed to remove the tablet from the die. The maximum force shown by the upper punch in the unlubricated case dropped from 1,450 Kg. to 1,250 Kg. in the lubricated case. The maximum force transmitted to the lower punch increased from 880 Kg. in the unlubricated case to 1,150 Kg. in the lubricated case.

Reproductivity of Measurements.—The data in Table I give some information on the reproductivity of force and displacement measurements with the instrumented machine. This table summarizes maximum force and displacement data for tablets made over a period of about six hours with no change in tablet machine force setting or settings of the sensitivity of the force and displacement strain gauge amplifiers. Compression time was varied as indicated in the table. In each case the die was hand charged with 570 mg. of a sulfathiazole granulation weighed to the nearest milligram. A $^{3}/_{8}$ inch flat-headed punch and die set was used in the machine.

Figure 3 gives additional information on the reproductivity of measurement with this machine. The two sets of points on the graph were obtained from punching two tablets under identical conditions except for punching speed. The oscillograph records were read and this plot resulted from the data obtained. In the measurement the speed of the tablet machine was adjusted so that the oscillograph record at each speed occupied exactly the same space on the chart. Tracings were made of the curves and overlaid. The two pairs of curves coincided exactly as nearly as could be seen with the eye. This indicated that in the range studied the force-displacement function was essentially independent of compression time.

TABLE I.—TYPICAL MAXIMUM FORCE AND DISPLACEMENT DATA

Tablet Number	Compression Time, Sec.[a]	Maximum Force Recorded, Kg.	Compression Distance, cm.[b]
100	95	1210	0.188
101	95	1205	0.184
102	95	1210	0.184
103	95	1210	0.184
104	95	1205	0.189
105	95	1210	0.189
106	95	1200	0.184
107	95	1200	0.178
108	95	1200	0.183
109	95	1200	0.179
110	50	1200	0.181
111	50	1200	0.182
112	50	1200	0.182
113	50	1190	0.182
114	50	1190	0.182
115	50	1200	0.181
116	50	1195	0.180
117	50	1195	0.183
118	50	1200	0.182
119	50	1190	0.181
120	32	1200	0.184
121	32	1195	0.181
122	32	1200	0.184
123	32	1195	0.181
124	32	1210	0.183
125	32	1200	0.182
126	32	1205	0.179
127	32	1190	0.179
128	32	1190	0.182
129	32	1190	0.181

[a] Elapsed time between exertion of first 50 Kg. of force and maximum force.
[b] From 50 Kg. to maximum force.

APPLICATIONS OF THE INSTRUMENTED MACHINE

Among the studies in progress or planned are included studies to determine compressional energies, the effect of tablet lubricants on the force differential between the upper and lower punches and hence the effectiveness of lubricants, the effect of the geometry of punch facings on this same quantity, the effect of punching speed on appropriate quantities and the effect of granulation variables. The instrumented machine is capable of assisting in studies of any phase of tablet compression where punching force and punch displacement information are needed.

Certain additional equipment is in the design stage which will permit force and displacement studies at higher punching speeds. The results of these investigations and constructions will be published later.

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(2) Higuchi, T., Rao, A. N., Busse, L. W., and Swintosky, J. V., *ibid.*, **42**, 194(1953).

Investigation of Some Complexes Formed in Solution by Caffeine*

IV. Interactions Between Caffeine and Sulfathiazole, Sulfadiazine, *p*-Aminobenzoic Acid, Benzocaine, Phenobarbital, and Barbital

By TAKERU HIGUCHI and JOHN L. LACH

This investigation is a continuation of previous studies made on the complexing behavior of caffeine with numerous pharmaceuticals. By means of solubility analysis it is shown that one-to-one complexes are formed between the xanthine and the pharmaceuticals listed under the title. The barbiturates appear to form both one-to-one and two-to-one complexes. The stability constants of the several complexes have also been estimated.

IN EARLIER PUBLICATIONS from these laboratories incontrovertible evidence of complex formation between caffeine and a number of pharmaceutical acids, esters, and salts was presented (1–3). It was shown that these complexes were distinct molecular species capable of existing in both liquid and solid phase in fixed molecular proportions. The present communication is concerned with further extension of these caffeine complexing studies to certain aromatic and nonaromatic nitrogenous compounds. Data are presented for complexes formed between caffeine sulfathiazole, sulfadiazine, *p*-aminobenzoic acid, benzocaine, phenobarbital, and barbital.

SULFONAMIDES

Although Neish in 1948 (4) observed that caffeine had a solubilizing effect on the sulfonamides, he made no attempt to study this interaction from a quantitative viewpoint. In the present investigation the stability constants of this complexing reaction have been tentatively evaluated for sulfathiazole and sulfadiazine.

Experimental

Reagents.—Recrystallized sulfathiazole U. S. P.; m. p. 201–202°; recrystallized sulfadiazine U. S. P.; m. p. 255–256°; recrystallized caffeine U. S. P., m. p. 235–237°; 5 *N* sulfuric acid; 0.1% sodium nitrite; 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride.

Procedure.—Excess quantities (75 mg. of sulfathiazole or 25 mg. of sulfadiazine) of sulfonamide were placed in 125-ml. glass-stoppered bottles together with varying but accurately weighed amounts of caffeine (0.0–1.5 Gm.) and 50-ml. portion of distilled water. The bottles were placed in a mechanical shaker in a constant temperature bath and equilibrated for eight hours at 30°. Aliquot portions of the supernatant liquid were removed and analyzed for the sulfonamide concentration at the end of this time The method of Bratton and Marshall (5) was used for determining both sulfathiazole and sulfadiazine.

Results and Discussion

In accordance with the findings of Neish, caffeine has a definite solubilizing action on the sulfonamides studied. As shown in Figs. 1 and 2, the amounts of both drugs in equilibrium with their respective solid phase increase progressively with caffeine concentration. Although we attribute this increase in the solubilities of the sulfonamides to complex formation, it is difficult to determine the exact stoichiometry of the reaction because of the high solubilities of the reaction products. Nevertheless it seems highly likely that these are one-to-one complexes since this type appears to be the dominant one. This assumption is partly borne out, furthermore, by the linear dependency of the reaction on caffeine concentration.

In a manner analogous to those employed in previous studies (3) it is possible to calculate the stability constants for the two postulated reactions:

$$\text{Sulfathiazole} + \text{Caffeine} \rightleftharpoons \text{Sulfathiazole-Caffeine} \quad (1)$$

and

$$\text{Sulfadiazine} + \text{Caffeine} \rightleftharpoons \text{Sulfadiazine-Caffeine} \quad (2)$$

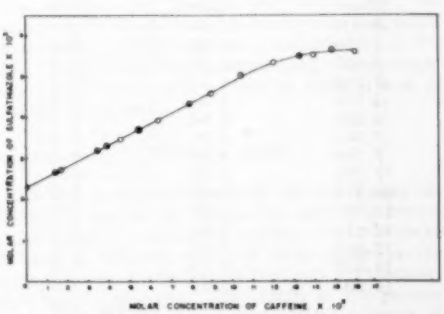

Fig. 1.—Influence of caffeine on the solubility of sulfathiazole in water at 30.0° C.

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TABLE I.—TOTAL SOLUBILITY OF SULFATHIAZOLE IN WATER CONTAINING CAFFEINE AT 30° C.

Caffeine Added to System Concn. × 10^3 Molar	Total Sulfathiazole at Saturation, Concn. × 10^3 Molar	Equilib. Const.
0.000	2.27	...
1.419	2.66	12.5
1.514	2.63	10.7
1.674	2.74	12.7
3.457	3.21	12.4
3.922	3.35	12.5
3.944	3.27	11.2
4.573	3.47	11.8
5.468	3.68	11.7
6.375	3.92	11.7
7.951	4.27	11.4
7.956	4.30	11.5
9.017	4.59	11.7
10.448	4.73	10.6
10.485	5.01	11.8
12.250	5.35	11.4
13.342	5.45	10.7
14.069	5.54	10.5
14.074	5.46	10.2
14.908	5.62	10.1
15.089	5.69	10.2
15.907	5.58	9.3
	Av.	11.3

Let X = Values column 1. Y = Values column 2.

According to Eq. (1):

$$K_c = \frac{(Y - 2.27 \times 10^{-3})}{(2.27 \times 10^{-3})[X - (Y - 2.27 \times 10^{-3})]}$$

These stability constants are tabulated in Tables I and II.

It is interesting to note that 1 Gm. of caffeine per 100 ml. of water increases the solubility of sulfathiazole 50%; sulfadiazine on the other hand is less readily solubilized. The rather low stability constants indicate that the interaction between the sulfonamides studied and caffeine is not too strong. Although the present study was limited to only two sulfonamides it is likely that other members of this class of drugs will behave similarly.

TABLE II.—TOTAL SOLUBILITY OF SULFADIAZINE IN WATER CONTAINING CAFFEINE AT 30° C.

Caffeine Added to System Concn. × 10^3 Molar	Total Sulfadiazine at Saturation, Concn. × 10^3 Molar	Equilib. Const.
0.000	3.64	...
1.454	3.80	3.0[a]
1.467	3.77	2.4[a]
3.716	4.51	6.5
3.728	4.50	6.4
5.285	4.84	6.3
5.964	5.04	6.5
7.356	5.32	6.3
7.703	5.55	6.9
10.470	6.47	7.5
10.742	6.36	7.0
12.410	6.64	6.7
12.865	7.04	7.3
14.556	7.69	7.9
14.957	7.51	7.1
	Av.	6.9

[a] These values omitted in obtaining the average.

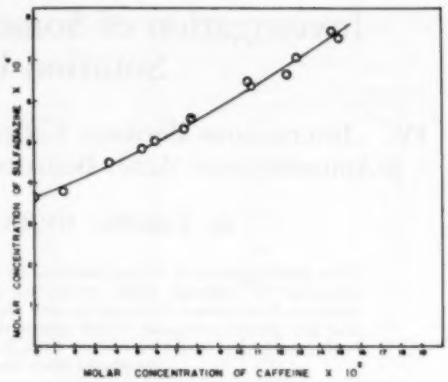

Fig. 2.—Influence of caffeine on the solubility of sulfadiazine in water at 30.0° C.

p-AMINOBENZOIC ACID

Although other investigations have indicated complex formation between hydroxy acids and caffeine, no previous studies of similar nature covering analogous amino acids have been made. Data presented herein indicate that a one-to-one complex is formed between *p*-aminobenzoic acid and caffeine.

Experimental

Reagents.—Purified *p*-aminobenzoic acid (PABA), Eastman Kodak Co., m. p. 187–188°; recrystallized caffeine U. S. P., m. p. 235–237°; 0.1 *M* dipotassium acid phosphate solution; 0.5 *N* sodium hydroxide solution.

Procedure.—An excess quantity (0.5 Gm.) of PABA, accurately weighed, was placed in 125-ml glass-stoppered bottles together with varying but accurately weighed amounts of caffeine (0.0–2.0 Gm.) and 50 ml. of distilled water added to each. The bottles were equilibrated for twenty-four hours in a constant temperature water bath at 30°. Aliquot portions were then removed and the solutions were analyzed.

The *p*-aminobenzoic acid contents of the samples were determined spectrophotometrically. The caffeine was first removed from the caffeine-PABA mixture by making the solution alkaline with 0.5 *N* sodium hydroxide and extracting with chloroform. The aqueous extract containing the sodium salt of *p*-aminobenzoic acid was diluted with buffer prior to spectrophotometric analysis at 266 mμ.

Results and Discussion

The effect of caffeine on the amount of PABA in solution in equilibrium with a definite quantity of the acid is shown in Fig. 3 and tabulated in Table III. It is evident that the overall reaction is relatively complex and closely resembles that between caffeine and salicylic acid (3). At low caffeine concentration a straight line relationship is obtained between the concentration of acid and caffeine in solution. This increase in solubility of the acid is due to complex formation (Eq. 3) and continues until the solubility limit of the complex is reached.

$$\text{PABA} + \text{Caffeine} \rightleftharpoons \text{PABA-Caffeine} \qquad (3)$$

TABLE III.—TOTAL SOLUBILITY OF *p*-AMINOBENZOIC ACID IN WATER CONTAINING CAFFEINE AT 30° C.

Caffeine Added to System Concn. × 10^2 Molar	Total Acid Concentration at Saturation × 10^2 Molar
0.000	4.579
0.460	4.873
0.506	4.926
0.581	4.978
0.993	5.312
1.051	5.294
1.337	5.510
1.640	5.557
1.901	5.548
2.116	5.554
2.323	5.539
2.548	5.556
3.229	5.434
3.337	5.259
3.692	5.092
4.614	4.260
5.341	3.594
6.590	2.919
8.591	2.244
10.651	2.016
11.831	1.963
13.662	2.069
15.961	2.209
17.060	2.279
18.266	2.406
19.599	2.498

Analogous to the caffeine-salicylic acid system point *S* in Fig. 3 represents the saturation point of the solution with respect to this complex. On further addition of caffeine the complex (Eq. 3) continues to form, but precipitates out of the already saturated system. The total concentration of PABA in solution is an invariant in this region as shown by the flat plateau, independent of the amount of caffeine, since any acid precipitated in the form of the complex is replaced by further dissolution of the solid free acid. At point *R*, the system is no longer saturated with respect to the acid, since all of the excess solid acid has been converted to the complex. Further addition of caffeine results in depletion of acid in solution. Formation of a higher complex, possibly two caffeine to one acid, probably is responsible for solubilization of the 1:1 complex at higher caffeine concentrations as indicated by *P*.

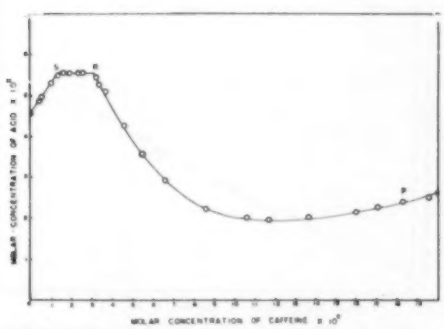

Fig. 3.—Phase diagram of PABA-caffeine system in water at 30.0° C. Molar concn. of solubilized *p*-aminobenzoic acid plotted against molar concn. of caffeine.

On the basis of a one-to-one relationship an average value of 48 was obtained for the equilibrium constant for reaction (3) over the straight line portion of the diagram, where

$$K_c = \frac{(\text{PABA} - \text{Caffeine})}{(\text{PABA})(\text{Caffeine})}$$

The value was calculated from experimental data in Table III in the following manner:

Let x = Values, column 1
y = Values, column 2
PABA-Caffeine = $y - 4.58 \times 10^{-2}$ moles/L.
PABA = 4.58×10^{-2} moles/L.
Caffeine = $x - (y - 4.58 \times 10^{-2}$ moles/L.)

The stoichiometric ratio of the components of the *p*-aminobenzoic acid-caffeine complex which was formed in the plateau region can be calculated from the phase diagram. This is possible because, if the above analysis is true, the amount of caffeine represented by the plateau is equal to that entering into the complex during this interval. The corresponding amount of the acid being converted to the complex is equal to that present in form of free undissolved solid at point *S*.

Thus,

Caffeine content of complex formed in the plateau region = 1.8×10^{-2} moles/L. (from diagram)

p-Aminobenzoic acid content = total acid − acid in solution at point *S*
$= 7.3 \times 10^{-2} - 5.5 \times 10^{-2}$
$= 1.8 \times 10^{-2}$ moles/L.

Then the stoichiometric ratio equals

$$\frac{\text{Caffeine content}}{\text{PABA content}} = \frac{1.8 \times 10^{-2}}{1.8 \times 10^{-2}} = 1$$

The results indicate that the complex is, in all probability, a one to one complex.

Further evidence of this relationship was obtained by direct chemical analysis of the complex. Addition of a hot saturated caffeine solution to a hot saturated PABA solution yielded a white precipitate after cooling. This was filtered and dried, m. p. 70–71°. Analysis of this precipitate gave a stoichiometric ratio of 0.99 with respect to caffeine and *p*-aminobenzoic acid.

BENZOCAINE

As with *p*-aminobenzoic acid, the complexing behavior of none of its derivatives, including benzocaine, appears to have been studied. Because of the presence of the polar ester and amino groups on the ring, however, a considerable tendency in this direction can be expected. The results of the present investigation show that a fairly stable complex of the one-to-one type is formed in solution.

Experimental

Reagents.—Purified benzocaine U. S. P., m. p. 90–91°; recrystallized caffeine U. S. P., m. p. 235–237°; 0.5 *N* sodium hydroxide solution; 0.1 *M* dipotassium acid phosphate solution.

Procedure.—Excess quantities (0.70 Gm.) of benzocaine were used per 50 ml. of distilled water together with varying but accurately weighed amounts of caffeine. Equilibration time was six hours at 30.0°. The analytical procedure employed for the determination of benzocaine was similar to that of *p*-aminobenzoic acid. The sodium *p*-aminobenzoate obtained after hydrolysis of the ester-caffeine mixture with sodium hydroxide was determined spectrophotometrically. The caffeine was first removed from the hydrolyzed solution by extraction with chloroform. The aqueous extract containing the sodium salt of PABA was diluted with buffer prior to spectrophotometric analysis at 266 mμ.

Results and Discussion

It is evident from the data presented in Table IV and Fig. 4 that the ester complexes rather strongly with caffeine. In the figure where saturation concentration of benzocaine has been plotted against total added caffeine it is apparent that this behavior can lead to a severalfold increase in the concentration of the surface anesthetic. Unlike the situation found in the caffeine-PABA system, however, the plateau found on the phase diagram was not due to precipitation of the complexes but rather to the limited solubility of caffeine itself, the solid phase in the system being composed of mixtures of solid caffeine and solid benzocaine.

The assumption of the following reaction appears to be in accord with the experimental data:

$$\text{Benzocaine} + \text{Caffeine} = \text{Benzocaine-Caffeine}$$

$$K_c = \frac{(\text{Benzocaine-Caffeine})}{(\text{Benzocaine})(\text{Caffeine})}$$

Since the total solubility of benzocaine was limited by the solubility of caffeine (13.3×10^{-2} moles/L. at 30.0°) no plateau region analogous to that found in the PABA-Caffeine system (*S–R* Fig. 3) appears in the phase diagram. This is due to the fact that the solution became saturated with respect to caffeine instead of the complex. The stoichiometry of the complexing reaction can be computed, however, from the increases in the solubilities of both benzocaine and caffeine resulting from the complex formation. This is possible because the molar quantities represented by the increases in the solubilities of both components of the system correspond directly to the amounts of the respective components entering into complex formation. Thus

$$\text{Solubility increase of caffeine} = 17.5 \times 10^{-2} - 13.3 \times 10^{-2} = 4.2 \pm 0.2 \times 10^{-2} \text{ moles/L.}$$

where 17.5×10^{-2} was the amount of caffeine added to the point of the break in the diagram and 13.3×10^{-2} the solubility of caffeine in pure water at 30°.

Solubility increase of benzocaine = Total Benzocaine − Benzocaine at 30° = $4.9 \times 10^{-2} - 0.5 \times 10^{-2} = 4.4 \times 10^{-2}$ moles/L. Then the stoichiometric ratio equals

TABLE IV.—TOTAL SOLUBILITY OF BENZOCAINE IN WATER CONTAINING CAFFEINE AT 30° C.

Caffeine Added to System Concn. × 10^2	Total Benzocaine at Saturation, Concn. × 10^2	Equilib. Const.
0.000	0.515	...
1.066	0.744	53.2
3.183	1.184	51.7
5.410	1.662	56.6
6.339	2.082	63.7
7.271	2.135	56.7
8.332	2.305	53.2
9.426	2.691	58.3
9.443	2.623	55.9
10.634	2.807	53.4
11.769	3.343	61.5
13.003	3.575	59.8
14.087	4.053	65.1
14.673	4.087	62.4
15.940	4.532	65.3
16.489	4.522	62.4
17.162	4.812	64.8
17.862	4.928	63.6
18.520	4.909	60.3
19.725	5.034	57.7
	Av.	59.1

$$\frac{\text{Caffeine content}}{\text{Benzocaine content}} = \frac{4.2 \times 10^{-2}}{4.4 \times 10^{-2}} = 0.95$$

The results indicate that the complex is of a one-to-one type. This is further substantiated by the fact that the equilibrium constant calculated on this basis over the concentration range studied remained fairly constant; 59.1 is the average value obtained.

PHENOBARBITAL AND BARBITAL

Although the interactions of the barbiturates with the xanthines have been previously studied, our present findings are discussed in some detail as they are at some variance with those reported by others. Higgins and Dunker (6) showed that phenobarbital and theophylline interact in the ratio of one phenobarbital molecule to two theophylline molecules to form a stable complex and that barbi-

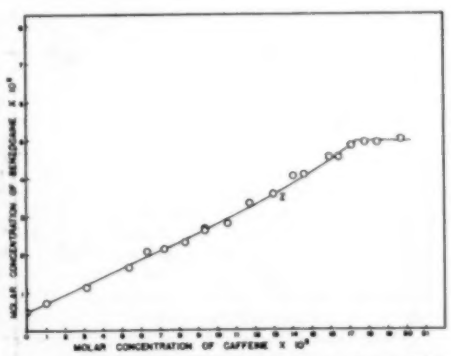

Fig. 4.—Phase diagram of benzocaine-caffeine system in water at 30.0° C. Molar concn. of solubilized benzocaine plotted against molar concn. of caffeine. Point Z on the diagram represents the solubility limit of caffeine.

TABLE V.—TOTAL SOLUBILITY OF BARBITAL IN WATER CONTAINING CAFFEINE AT 30° C.

Caffeine Added to System Concn. × 10^2 Molar	Total Barbital at Saturation Concn. × 10^2 Molar
0.000	4.408
0.968	4.461
2.110	4.541
3.265	4.615
3.972	4.647
4.696	4.647
5.830	4.647
6.615	4.621
7.869	4.602
8.551	4.556
10.402	4.190
12.698	3.914
15.881	3.731
17.666	3.670

turic acid and theophylline react to give a 1:1 complex. According to these investigators, however, phenobarbital does not react with either caffeine or theobromine. The results of the present investigation, however, indicate that phenobarbital as well as barbital, the only other barbiturate studied, complexes with caffeine under the conditions employed.

Experimental

Reagents.—Recrystallized phenobarbital U. S. P., m. p. 174–175°; recrystallized barbital U. S. P., m. p. 189.5–190.5°; recrystallized caffeine U. S. P., m. p. 235–237°; 0.1 *N* alcoholic sodium hydroxide; thymolphthalein and alizarin yellow as indicators.

Procedure.—Excess quantities of phenobarbital were used, 400 mg. per 50 ml. distilled water at 30° and 250 mg. at 15°. For barbital 1.20 Gm. at 30° and 0.80 Gm. at 15°. Aliquot portions were analyzed after an equilibration time of six hours.

Phenobarbital and barbital were determined by microtitration with alcoholic sodium hydroxide (7), with thymolphthalein as the indicator for phenobarbital and alizarin yellow for barbital.

Results and Discussion

The effect of varying caffeine concentration on the apparent solubilities of barbital and phenobarbital is shown in Figs. 5–6, and tabulated in Tables V–VIII. The phase diagrams obtained here are

TABLE VI.—TOTAL SOLUBILITY OF BARBITAL IN WATER CONTAINING CAFFEINE AT 15° C.

Caffeine Added to System Concn. × 10^2 Molar	Total Barbital at Saturation Concn. × 10^2 Molar
0.000	3.187
0.644	3.203
1.190	3.252
1.727	3.245
2.511	3.278
3.556	3.274
4.640	3.182
6.155	2.777
7.220	2.623
9.758	2.438
12.556	2.341
15.181	2.341

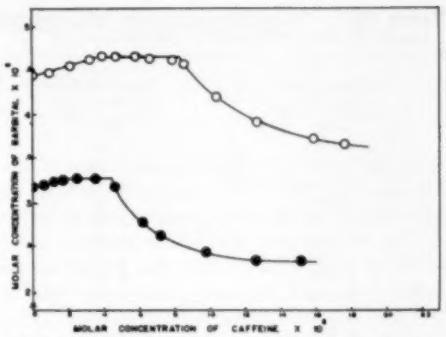

Fig. 5.—Phase diagram of barbital-caffeine system in water at 30.0° C. Molar concn. of solubilized barbital plotted against molar concn. of caffeine. A complex of the 2:1 type (two barbital molecules to one caffeine molecule) was obtained at 30.0° C. and at 15.0° C. ○, 30° C. ●, 15° C.

TABLE VII.—TOTAL SOLUBILITY OF PHENOBARBITAL IN WATER CONTAINING CAFFEINE AT 30° C.

Caffeine Added to System Concn. × 10^2 Molar	Total Phenobarbital at Saturation Concn. × 10^2 Molar
0.000	6.31
1.044	6.42
1.524	6.58
2.075	6.61
2.232	6.84
2.642	6.77
3.491	6.90
4.132	6.92
4.676	6.84
5.061	6.84
5.232	5.28
5.402	5.81
5.901	5.02
6.489	5.02
7.482	4.55
8.337	4.45
8.883	4.49
9.756	4.49

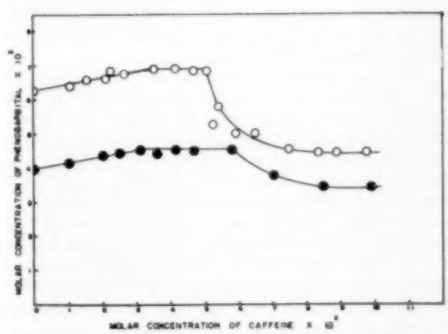

Fig. 6.—Phase diagram of phenobarbital-caffeine system in water at 30.0° C. Molar concn. of solubilized phenobarbital plotted against molar concn. of caffeine. A 2:1 complex (two phenobarbital molecules to one caffeine molecule) was obtained at 30.0° C. and a 1:1 type at 15.0° C. ○, 30° C. ●, 15° C.

TABLE VIII.—TOTAL SOLUBILITY OF PHENOBARBITAL IN WATER CONTAINING CAFFEINE AT 15° C.

Caffeine Added to System Concn. × 10^3 Molar	Total Phenobarbital at Saturation Concn. × 10^3 Molar
0.000	3.98
1.010	4.14
2.019	4.37
2.534	4.44
3.115	4.53
3.630	4.42
4.145	4.50
4.676	4.50
5.796	4.50
7.014	3.80
8.486	3.46
9.903	3.40

analogous to that of the PABA-caffeine system. At low caffeine concentrations a straight line relationship is obtained between the concentration of barbiturate and caffeine in solution. This increase in solubility of the barbiturate with caffeine concentration is due to complex formation and continues until the solubility limit of the complex is reached.

$$\text{Barbiturate} + \text{Caffeine} \rightleftharpoons \text{Barbiturate-Caffeine} \quad (4)$$

As with the PABA-caffeine system the beginning of the flat plateau region of the phase diagram represents the saturation point of the solution with respect to this complex and the barbiturate. Although the complex (Eq. 4) continues to form with addition of caffeine, it precipitates out of the already saturated system. The total concentration of barbiturate in solution remains unchanged in the plateau region of the diagram and is independent of the amount of caffeine since any barbiturate precipitated in form of the complex is replaced by further dissolution of solid free barbiturate until all of the solid free barbiturate has been converted to the complex. At this point further addition of caffeine to the system results in depletion of barbiturate in solution as shown by the break in the plateau region of the diagram.

The stoichiometric ratio of the components of the barbiturate-caffeine complex formed in the plateau region can be calculated from the phase diagram in a manner analogous to that of the PABA-caffeine system As previously pointed out this is possible because, if the above analysis is true, the amount of caffeine represented by the plateau is equal to that entering into the complex during this interval. The corresponding amount of the barbiturate being converted to the complex is equal to that present in form of free undissolved solid present in the system at the beginning of this plateau region.

Thus for barbital at 30°,

Caffeine content of complex formed in the plateau region = $8.3 \times 10^{-3} - 3.9 \times 10^{-3} = 4.4 \pm 0.2 \times 10^{-3}$ moles/L. (from diagram)

Barbital content = total barbital − barbital in solution at beginning of plateau = $13.0 \times 10^{-3} - 4.6 \times 10^{-3} = 8.4 \times 10^{-3}$ moles/L.

Then the stoichiometric ratio equals

$$\frac{\text{Caffeine content}}{\text{Barbital content}} = \frac{4.4 \times 10^{-3}}{8.4 \times 10^{-3}} = 0.52$$

In other words, one caffeine molecule combines with two barbital molecules. At 15° a ratio of 0.48 was obtained. The results indicate that the complex is of the 2:1 type. Chemical analysis of the precipitated complex, m. p. 145–147°, gave a stoichiometric ratio of 0.50.

Quantitative analysis of the phase diagram for phenobarbital at 30° gave a ratio of 0.54 with respect to caffeine and phenobarbital. A value of 0.52 was obtained for the stoichiometric ratio after chemical analysis of the precipitated complex, m. p. 145–146°. The results indicate that the complex is of the 2:1 type, two molecules of phenobarbital complexing with one molecule of caffeine.

However, at 15° analysis of the phenobarbital-caffeine phase diagram gave a stoichiometric ratio of 1.06; the ratio based on chemical analysis of the precipitated complex was 1.04. The results indicate a 1:1 type of complex This one-to-one complex obtained at 15° can be rationalized on the basis that at 30° this complex is too soluble to permit isolation whereas at 15° it is less soluble than the 2:1 type and precipitates out of solution.

As pointed out earlier, Higgins and Dunker concluded that, under their experimental conditions, phenobarbital did not react with caffeine to form a complex Present results indicate that the reaction occurs. The reason for this apparent discrepancy probably rests in the fact that the method of study of complex formation employed in the present instance was considerably more sensitive to such phenomena and that it did not depend on isolation of these relatively unstable products. The very sensitive nature of these molecular compounds and their phase relationship is evident in the fact that only the 2:1 type of phenobarbital-caffeine is recoverable at 30° whereas at 15° the same system yields only a 1:1 complex.

It is rather likely that many of the systems studied by Higgins and Dunker may yield positive evidence of molecular complex formation when examined by the present method.

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The Assay of Digoxin Preparations*

By DANIEL BANES

A method is proposed for the determination of digoxin, based upon the color developed with *m*-dinitrobenzene in weakly alkaline solution. Gitoxin does not interfere when present in concentrations lower than 8 per cent.

THE PRESCRIBED ASSAY method for digoxin preparations official in U. S. P. XIV (1) depends upon the blue color produced when *m*-dinitrobenzene reacts with the butenolide ring in the presence of strong alkali. The color is developed at 0° to 2°, and its absorbancy is measured in the spectrophotometer exactly five minutes after the addition of the alkali. But at 0° under the conditions specified, the maximum intensity is achieved in less than five minutes, and the color then fades rapidly. At higher temperatures these reactions are accelerated. It is therefore difficult to obtain reproducible absorbancies for aliquots of a standard digoxin solution treated in an identical manner. Moreover, gitoxin, which occurs as a concomitant glycoside, yields a similar blue color in the reaction. The U. S. P. provides a purity test to limit the permissible concentration of gitoxin by the requirement that 1 mg. of digoxin, dissolved in acetic acid containing iron, and underlaid with sulfuric acid shall produce "a brown ring free from red...at the junction of the two liquids." But Pesez (2) has shown that the red color developed by significant quantities of gitoxin may be masked by the yellow-brown colors produced by other pure glycosides. The test is even more unreliable in the presence of tablet extractives which may be charred by sulfuric acid. For these reasons it seemed desirable to seek a more specific and more reliable procedure for the determination of digoxin.

Hassall and Lippman (3) have treated various glycosides with *m*-dinitrobenzene in faintly alkaline solutions, and they have obtained red-violet products more stable than the blue color studied by Raymond (4), Canbäck (5), and Anderson and Chen (6). It was found in this laboratory that digoxin slowly developed a blue-violet color with a maximum at 615 mμ when treated with *m*-dinitrobenzene in dilute alkali. Under these conditions, gitoxin yielded a feeble red-violet color which faded rapidly, and its absorbancy was less than a twelfth of the absorbancy due to digoxin at 615 mμ. Since the concentration of the alkali was found to be a critical factor in the color development, it was necessary to exclude acidic interferences such as stearic acid by means of the extraction procedure described below. When samples of pure digoxin were subjected to this extraction, recoveries ranged from 99.5 to 100.3 per cent.

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EXPERIMENTAL

Materials.—*Solvents.*—Ethanol, chloroform, isooctane of U. S. P. grade.

Reagents.—(*a*) Keller-Kiliani reagent; 60 ml. of glacial acetic acid was mixed with 1 ml. of 9% ferric chloride solution and 5 ml. of concentrated sulfuric acid.

(*b*) Alkaline *m*-dinitrobenzene reagent; 1.6 Gm. of crystalline *m*-dinitrobenzene was dissolved in 80 ml. of hot alcohol. The mixture was cooled, 1.0 ml. of tetramethylammonium hydroxide solution (10% in water) was added, and the solution was diluted to 100 ml. with alcohol.

(*c*) Windaus-Schwarte reagent; 250 ml. of concentrated sulfuric acid was mixed with 0.1 ml. of 9% ferric chloride solution.

Crystalline Glycosides.—U. S. P. Reference Standard Digoxin and crystalline gitoxin were used as received from the U. S. P. Revision Committee. Solutions in alcohol were prepared to contain 50 μg. of glycoside per ml. of solution.

Spectrophotometer.—A Beckman DU quartz spectrophotometer, with matched 1 cm. Corex cells, was used.

Chromatographic Tube.—Equipment used has been described in a previous publication (7).

Preparation of Samples.—(*a*) *Injection.*—An accurately measured volume of digoxin injection equivalent to 2.5 mg. of the glycoside was transferred to a separator containing about 50 ml. of 10% sodium sulfate solution and 2 ml. of approximately normal hydrochloric acid solution. The mixture was extracted with seven 25-ml. portions of chloroform. Each chloroform extract was washed in a second separator containing 10 ml. of water, and filtered through a pledget of cotton into a beaker. The combined chloroform extracts were evaporated to dryness, and the residue was dissolved in hot alcohol. The solution was cooled, washed into a 50-ml. volumetric flask, and diluted to exactly 50 ml. with alcohol.

(*b*) *Tablets.*—A counted number of tablets were ground to a fine powder and a quantity of powder equivalent to 2.5 mg. of digoxin was mixed in a beaker with 20 ml. of alcohol. The beaker was covered with a watch glass and the mixture was heated to simmering for twenty minutes, with frequent stirring. The warm mixture was washed into a 250-ml. separator with three 10-ml. portions of alcohol and 50 ml. of water. After thorough

TABLE I.—THE DETERMINATION OF DIGOXIN AND GITOXIN

Sample	Description	Recovery by Proposed Method: Digoxin	Gitoxin	Total	Digitoxosides by Keller-Kiliani Test
1	2.5 mg. digoxin plus 0.05 mg. gitoxin	2.55 mg.	0.057 mg.	2.61 mg.	2.54 mg.
2	2.5 mg. digoxin plus 0.10 mg. gitoxin	2.48	0.108	2.59	2.56
3	2.5 mg. digoxin plus 0.15 mg. gitoxin	2.48	0.154	2.63	2.57
4	2.5 mg. digoxin plus 0.20 mg. gitoxin	2.44	0.193	2.63	2.63
5	2.5 mg. digoxin plus 0.25 mg. gitoxin	2.41	0.234	2.65	2.70
6	2.5 mg. digoxin plus 2.50 mg. gitoxin	1.60	..	..	..
7	Injection,[a] 0.5 mg./ml.	0.479 mg./ml.	0.012 mg./ml.	0.491 mg./ml.	0.492 mg./ml.
8	Tablets,[b] 0.25 mg./tablet	0.231 mg./tab.	0.013 mg./tab.	0.244 mg./tab.	0.240 mg./tab.
9	Tablets, 0.25 mg./tablet	0.224 mg./tab.	0.012 mg./tab.	0.236 mg./tab.	0.234 mg./tab.
10	U. S. P. Digoxin Reference Standard	....	2.4%	....	....

[a] Assays by U. S. P. XIV procedure ranged from 0.439 to 0.472 mg./ml.
[b] Assays by U. S. P. XIV procedure ranged from 0.209 to 0.295 mg./tablet.

cooling the mixture was acidified with 1 ml. of approximately normal hydrochloric acid solution and immediately extracted with 50 ml. of isooctane. The aqueous layer was transferred to another separator, extracted again with 50 ml. of isooctane, and transferred to a 500-ml. separator. The isooctane-layers were extracted with one 20-ml. portion of 50% alcohol and two 20-ml. portions of water, and the washings were collected in the 500-ml. separator. The combined aqueous extracts were diluted to about 400 ml. with 10% sodium sulfate, and treated as described under "Injections" beginning with the words "The mixture was extracted...."

Colorimetric Determination of Digoxin.—A 5-ml. aliquot of the prepared sample solution and a 5-ml. aliquot of standard digoxin solution were each transferred to test tubes containing silicon carbide chips and were evaporated in the steam bath. The tubes were cooled in a vacuum desiccator for thirty minutes. Exactly 4.0 ml. of freshly prepared alkaline *m*-dinitrobenzene was added to each residue and the tubes were permitted to stand at room temperature for fifteen minutes with frequent shaking. The absorbancies of the developing blue-violet colors were then measured at 615 mμ relative to the reagent as blank until the maximum values were attained. The digoxin content of the sample was calculated by comparison with the standard.

The Determination of Gitoxin in the Presence of Digoxin.—A portion of the prepared solution equivalent to 1 mg. of digoxin (20 ml.) was evaporated to dryness. The residue was treated with 2.0 ml. of alcoholic hydrochloric acid solution (1 + 1) and permitted to stand at room temperature for sixty minutes with occasional stirring. The yellow solution was mixed with 10 ml. of 50% alcohol and the mixture was heated to gentle simmering for thirty minutes. Alcohol was added in small portions to maintain a volume of 10–12 ml. After cooling, the mixture was washed into a separator with 100 ml. of water and extracted with three 20-ml. portions of chloroform. The chloroform extracts were each washed with 10 ml. of water in a second separator and filtered through cotton into a beaker. The combined extracts were evaporated to dryness. The residue was heated with 5 ml. of isooctane and the cooled turbid mixture was chromatographed on a 10-Gm. Celite column, with 5.0 ml. of 50% v/v alcohol as immobile solvent and isooctane as mobile solvent. The preparation of such columns has been described in detail in a previous paper (7). Isooctane used in preparing the column was discarded and fresh solvent was used in chromatographic development. A forerun of 50 ml. was rejected, and the next 70-ml. portion of eluate was evaporated to dryness. The beaker was cooled and the residue was dissolved in 4.0 ml. of Windaus-Schwarte reagent. A 1.0-ml. aliquot of standard gitoxin solution was similarly evaporated and treated with the reagent. The pink colors were measured at 530 mμ relative to the reagent, and the maximum absorbancies were used to compute the gitoxin content of the sample.

DISCUSSION

The conditions selected for the reaction between digoxin and alkaline *m*-dinitrobenzene were influenced by the speed of color development, the stability and reproducibility of the color, the behavior of other glycosides in the reaction, and the solubility of *m*-dinitrobenzene. In the procedure described the maximum absorbancy was attained in 25–30 minutes at temperatures of 22–25°, and the readings remained within 2% of the maximum for five minutes. A series of nine standards yielded colors whose maximum absorbancies fell within ±1% of the mean value. There was only a slight deviation from Beer's law for quantities of digoxin between 100 and 300 μg. and a single standard was employed for colorimetric comparisons.

The concentration of alkali in the reaction mixture was found to be critical. Substances which consumed alkali interfered with color development. Even excessive quantities of gitoxin led to low color intensities, and it was necessary to devise a reliable test for gitoxin in the presence of digoxin. The procedure developed was based upon the work of Windaus and Schwarte (8), McChesney, *et al.* (9), Pesez (2), and Bellet (10). It depended upon the conversion of gitoxin to dianhydrogitoxigenin, and the chromatographic isolation of the latter steroid. A fluorimetric method similar to that proposed by Pesez (2) for the determination of gitoxin in samples of digitoxin may be preferable for rapid analyses.

A series of mixtures containing known quantities of digoxin and gitoxin were analyzed according to the procedures described above. The results (presented in Table I, Samples 1–6) showed that errors in the digoxin determination were less than 2.5% when the proportion of gitoxin was less than 8%. They indicated a high degree of accuracy in the estimation of both digoxin and gitoxin.

A sample of digoxin injection, and two samples of digoxin tablets were analyzed for digoxin and gitoxin by the proposed methods. U. S. P. Reference Standard Digoxin was also examined for gitoxin content. Results of these analyses (Table I, Samples 7–10) indicated that the methods were applicable to the assay of commercial digoxin preparations. The total recoveries for digoxin plus gitoxin were in agreement with values obtained when the Keller-Kiliani test was applied to the prepared solutions for the estimation of total digitoxosides, using digoxin as standard.

SUMMARY

1. A convenient, reliable colorimetric test has been proposed for the determination of digoxin, using *m*-dinitrobenzene in weakly alkaline solution as the reagent.

2. A procedure has been described for the colorimetric estimation of gitoxin in the presence of digoxin.

3. The tests are applicable to the assay of commercial digoxin preparations.

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A Method of Evaluating Enteric Coatings Using the White Rat*

By F. C. HAMMERNESS† and C. H. WALDON‡

A method of testing enteric coatings based on enzymatic disintegration in the gastrointestinal tract of the white rat has been devised. Pills containing a dye were given to a group of rats, and the time between ingestion and detection of the dye in the urine was noted. After a waiting period, enteric coated pills containing the dye were administered and the time between ingestion and detection of the dye in the urine again noted. If either the dye was not detected or the elapsed time was comparable to the blank, the coating was considered unsatisfactory. The coating was considered satisfactory if the dye was noted after a reasonably increased lapsed time. The coatings used were cellulose acetate hydrogen phthalate and cellulose acetate stearate. The dye used was Pyridium®.

THERE HAVE BEEN many and varied methods developed (1–12) to test enteric coated tablets and pills. Most of the methods were based on timed disintegration in artificial gastric juices. It has been shown by Bauer and Masucci (13) that natural digestive action on cellulose derivatives is indicated. On this basis an *in vivo* method is presented.

EXPERIMENTAL

Preliminary Investigation.—The coating was accomplished in the conventional manner with a small bowl with a diameter of 4 inches as the coating pan. The coatings were applied with a small paint sprayer and dried with the aid of a hand hair dryer.

The formulas developed for the experiment are:

I.	Cellulose acetate hydrogen phthalate	10.0
	Ethyl acetate	50.0
	Alcohol, 95%	50.0
II.	Cellulose acetate stearate	20.0
	Ethyl acetate	75.0
	Alcohol, 95%	75.0

The Experiment.—Ten human subjects and ten white rats of the Denver variety, Wistar strain, weighing approximately 250 Gm. were selected at random. No alterations were made in the respective diets of either group.

The rat was used as a method of determining the efficiency of various enteric coatings. If the method

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was to be of value in interpreting the results obtained on the rats, it must be correlated to those obtainable on human subjects. To accomplish this, similar experiments were performed on both human subjects and rats alike.

In determining comparative kidney function and average excretion time, the subjects were given pills or tablets made in the laboratory, which contained about 5 mg. of phenylazodiaminopyridine hydrochloride[1] in each. Pills approximately 4 mm. in diameter were administered orally to the rats while under light anesthesia. The average excretion rate for humans was about 4.73 hours and for rats 1.88 hours. This was determined by noting the excretions of the rats at fifteen-minute intervals on filter paper, which gave an orange color. The human subjects were asked to report the time when they first noted an orange color in the urine. After a period of three days, similar pills and tablets coated with cellulose acetate hydrogen phthalate were administered. The average elapsed time before detection of the dye in the urine was 7.7 hours in the humans and 5.1 hours in the rats (Table I).

TABLE I.—EXCRETION RATE OF DYE IN HOURS OF TOTAL ELAPSED TIME

Human Subject	Uncoated	Coated	Rat	Uncoated	Coated
DBL	6:15	10:30	1	1:30	9:00
MS	4:45	5:30	2	1:00	4:00
JHW	4:45	8:00	3	2:15	5:00
RTC	5:30	8:00	4	2:10	4:00
CFS	3:15	8:30	5	2:05	3:00
HLJ	4:00	6:30	6	2:00	4:00
BIS	6:15	8:00	7	1:40	5:00
EHD	5:45	8:45	8	1:05	4:00
HUS	3:00	6:00	9	2:30	9:00
WEC	3:45	7:00	10	2:35	4:00
Av., Hr.	4.73	7.7		1.88	5.1

When the dye was detected in the urine after the coated pills or tablets had been given, it was evident that the coating had disintegrated. Due to the increase in the average elapsed time of the coated as compared to the uncoated pills or tablets, it would seem that disintegration had taken place somewhere beyond the stomach. To determine the site of disintegration, both groups were given tablets or pills of barium sulfate coated with cellulose acetate hydrogen phthalate, which could be detected by X-ray. The first X-ray was taken about two hours prior to the average elapsed time of the dye. A second or third picture was taken if necessary until results were obtained. In both the human and the rat, the coating disintegrated in the intestinal tract.

The same procedure was used with cellulose acetate stearate coating. Negative results were obtained with the dye although X-ray photographs showed that the coated tablets and pills had passed into the large intestine intact with one exception in each group. In one human subject no trace was found after four pictures, thus it was assumed that the tablet had passed on, and in one rat the pill was found still intact in the intestine after nine hours.

[1] Pyridium®, a trademark of Merck & Company, Inc.

SUMMARY AND CONCLUSION

The procedure for testing an enteric coating using the white rat consists of the use of a group of normal white rats sustained on a normal diet. Pills containing a suitable dye are given and the time noted when the dye first appears in the urine. After three days, the time needed for complete excretion of the dye, the rats are given the same pill with the enteric coating. Again the time is noted. If the dye is detected approximately three hours after the time noted for the blank, the coating is considered satisfactory, as it disintegrated beyond the stomach.

A method of testing enteric coatings based on natural action in the gastrointestinal tract of the white rat is proposed. The method is easily performed *in vivo*. The method gives satisfactory results and can be employed as a quick means of eliminating unsatisfactory coatings in experimental work in the development of new enteric coatings.

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The Thromboplastin Activity of Bovine Thoracic Aorta*

By LOUDON L. CAMPBELL†

Material was extracted from bovine thoracic aorta; this material possessed thromboplastin activity when employed in a Quick one-stage procedure for determining prothrombin time. By separating the aorta into its anatomical layers, intima, media, and adventitia, and extracting these individually, it was found that qualitative differences in thromboplastin activity existed between the three layers. When the three extracts of the layers were tested by the Quick one-stage procedure for the determination of prothrombin time, the intima possessed the least thromboplastin activity, the media the next most activity, and the adventitia the greatest thromboplastin activity. It is believed that this gradient in thromboplastin activity in the three layers of the aorta manifests the operation of homeostatic mechanism. As a source of thromboplastin, bovine thoracic aortic extract is superior to thromboplastin obtained from either bovine lung or rabbit brain.

IN THE SEARCH for thromboplastin activity most investigators go to tissues far removed from the location of the clot. Almost every tissue in the animal body possesses thromboplastin activity (1). However, rabbit brain and bovine lung are the richest sources of thromboplastin and are the tissues most often used for this purpose (1). While extracting thromboplastin from bovine lung by the method of Chargaff, this investigator subjected the bovine thoracic aorta to the same extraction procedure (2–6). By the quick one-stage procedure for determination of prothrombin time, the thoracic aorta yielded thromboplastic activity of comparable potency to that of bovine lung (7). Since the blood vessel is more intimately involved in the coagulation process than either the brain or the lung, the matter merited further investigation.

EXPERIMENTAL

The aorta was separated into intima, media, and adventitia, and each of these tissues extracted for thromboplastin. It was found that the adventitia was richest in thromboplastin activity; the media possessed the next greatest amount of activity, and the intima the least activity.

Homeostasis.—Such a gradient in concentration of thromboplastic activity in the layers of the aorta suggests a manifestation of the homeostatic mechanism. A break in the blood vessel wall constitutes a hemorrhagic threat to the life of the organism. This may be the reason for a greater concentration of thromboplastin activity in the adventitia. On the other hand, a break in the intima releases less thromboplastin so that clot formation is not as likely as it is in the case of the adventitia. Thromboplastin activity may be less concentrated in the intima so as to minimize thrombus formation and subsequent emboli.

Factors Favoring Clot (Thrombus Formation).—Thromboplastic activity seems to be concentrated in the adventitia, or else there is less inhibition of thromboplastin activity and greater acceleration of the release of thromboplastin activity here.

Factors Opposed to Clot (Thrombus Formation).—There appears to be less thromboplastin activity in the intima, or more inhibition of thromboplastin formation and a decrease in the rate of release of thromboplastin activity.

Apparently, the intima is rich in antithromboplastin, and the adventitia is poor in this inhibitor. Or it may mean that for some other reason the release of thromboplastin is more rapid from the adventitia than is its release from the media and intima.

Commercial Laboratory Source of Thromboplastin.—The bovine thoracic aorta may be an excellent commercial source for thromboplastin. It is much easier to handle than the bulky lungs of the cow; and gleaning the associated aortic fat is far less trouble than stripping the meninges and blood vessels of brain. Furthermore, the material has the same activity as that from either of these other sources and can be obtained less expensively. The extracted material is clear and does not possess suspended material as other thromboplastin preparations do. Nor is thoracic aortic thromboplastic extract contaminated with blood elements. Bovine lung, unless carefully perfused, usually contains blood elements (blood cells and plasma constituents).

PROCEDURE

In this coagulation study, 2 L. of oxalated beef plasma were used; the beef plasma was quick-frozen and stored at 5°. A stock preparation of beef lung thromboplastin kept at the same temperature was used to get a standard coagulation time. This material contained 0.5% phenol as a preservative. Using the Quick one-stage procedure for determination of prothrombin time, the beef lung thromboplastin and beef plasma gave an average prothrombin time of 20.0 seconds. This average was obtained on 100 clots.

Intact Thoracic Aorta.—Each of the 10 thoracic aortas were treated in the following manner: The aorta was stripped of fat and ground in an ordinary meat grinder; the average aorta weighed 25.0

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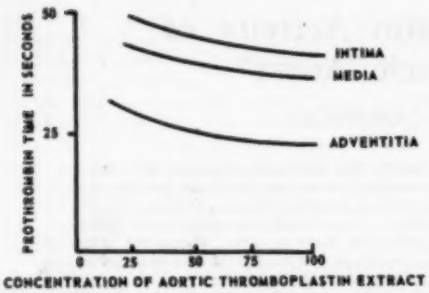

Fig. 1.—Differences in concentration of thromboplastin in intima, media, and adventitia.

Gm. An equal weight of cold normal physiological saline was added and the mixture was allowed to stand twelve hours at 5°. The solution was then filtered through gauze and centrifuged at 2,000 r. p. m. for fifteen minutes. The resulting clear supernatant contained the thromboplastin that was used in the Quick one-stage procedure for determination of prothrombin time; in 100 clots, the average prothrombin time was 19.1 seconds. Compared to this, the stock thromboplastin gave an average prothrombin time of 20.0 seconds. Bovine thoracic aortic extract lost its thromboplastic activity after one week; however, by adding 0.5% phenol to the solution its stability was maintained. Table I lists 10 aortic extracts, and their average prothrombin times.

TABLE I.—AVERAGE PROTHROMBIN TIMES FOR 10 THROMBOPLASTIN THORACIC AORTIC EXTRACTS

Number Aortas	Time, Seconds/100 Clots[a]	
1	19.1	(20.0)
2	19.0	(22.0)
3	18.9	(20.0)
4	19.0	(21.0)
5	19.0	(20.0)
6	19.3	(20.0)
7	19.2	(20.0)
8	19.4	(20.0)
9	20.0	(22.0)
10	19.0	(20.0)

[a] The figures in parentheses represent the average prothrombin time in seconds for the stock solution of thromboplastin.

Divided Thoracic Aorta.—The bovine thoracic aorta was easily separated into its three anatomical layers. The tubular aorta was cut with dissecting scissors so as to divide it along its length where the branches came off; the aorta was pressed and flattened out. The edge of the aorta on its smooth, glistening, intimal surface was picked up with tissue forceps and peeled free from the media. The same procedure was used in separating the adventitia; the edge of the aorta on the adventitial surface was picked up with the tissue forceps and pulled free from the media. The intima appeared to be almost tissue-paper thin; weights for it varied from 3.0 to 5.0 Gm. The adventitia felt and looked like a piece of cloth; weights for it varied from 8.0 to 11.0 Gm. The media was friable and spongy to the touch; weights for it varied from 10.0 to 12.0 Gm.

By this method five thoracic aortas were separated into intima, media, and adventitia; the average weights for each of these layers were, respectively, 4.0 Gm., 11.0 Gm., and 10.0 Gm. These three portions of aorta were treated individually in the manner described for the extraction of thromboplastin from the whole thoracic aorta. The average prothrombin times were: intima, 40.0 seconds; media, 36.0 seconds; and adventitia, 22.0 seconds. By dilution with normal physiological saline a difference in the thromboplastin activity of each of these layers could be shown. Figure 1 clearly depicts the differences in concentration of thromboplastin in each of the three layers.

SUMMARY AND CONCLUSIONS

1. The discovery that there exists a gradient in the concentration of thromboplastin activity in the bovine thoracic aortic wall—activity increasing as the periphery is approached—suggests that this is a defensive mechanism for maintaining constancy of the internal environment.

2. The thoracic aorta of the cow offers a new laboratory and commercial source for thromboplastin; it is much easier to handle than the commonly used beef lung and the expensive rabbit brain.

3. After centrifugation the aortic extract does not contain the objectionable suspended material as do the other thromboplastin sources.

4. Bovine thoracic aorta is inexpensive.

5. Bovine lung thromboplastin extract is usually contaminated with blood elements (blood cells and plasma constituents), unless carefully perfused; bovine thoracic aortic extract does not contain these elements.

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Absorption and Metabolism of Rutin and Quercetin in the Rabbit*

By CHARLES W. MURRAY, ALBERT N. BOOTH, FLOYD DeEDS, and FRANCIS T. JONES

The metabolic fate of orally administered rutin or its aglycone, quercetin, has been investigated in rabbits. Evidence for the absorption and metabolism of these compounds is based upon the urinary excretion of degradation products. One of these products has been isolated and identified as 3,4-dihydroxyphenylacetic acid. The amount isolated accounts for at least 25 per cent of the quercetin administered.

RUTIN, the rhamno-glucoside of quercetin, is being used extensively for therapeutic purposes, alone and in a variety of pharmaceutical formulations. The clinical use of rutin was announced first by Griffith, Lindauer, and Couch (1) in 1943. The dosage of rutin continues to be empirical, rather than based on a knowledge of its absorption, rates, and pathways of excretion, and the levels of rutin or its metabolites in the body fluids and tissues required to produce physiological effects. Realizing the need for such information, we have been studying the metabolic fate of rutin. The present paper is concerned with the isolation and identification of one of the metabolites of rutin and its aglycone, quercetin, in the urine of rabbits after oral administration of these compounds.

The literature dealing with absorption, metabolism, and excretion of orally administered rutin reveals marked disagreement among investigators. Rutin was given orally to dogs by Garino (2), and to rabbits by Kono (3) and Fukuda (4), all of whom reported the excretion of unchanged rutin in the urine. Field and Rekers (5) working with dogs could not confirm Garino. Porter, Dickel, and Couch (6) found only traces of rutin in human urine after oral administration. Couch Krewson, and Naghski (7) reported traces of rutin in the urine of rabbits after oral administration, but found 12.5 per cent of intravenously administered rutin excreted in the urine. Clark and MacKay (8) gave rutin orally to humans and rats and found practically none in the urine of either species. Ozawa (9) gave the closely related compound, 3′,4′-dihydroxyflavonol to rabbits and recovered less than one-tenth of the amount given from the urine and feces. However, by paper chromatography he showed that the urine contained three substances of different R_f values and concluded that they were derived from the parent substance by physiological processes. Kirtley and Peck (10) gave large doses of the related flavonoid hesperidin methyl chalcone to humans orally and failed to find the compound excreted in the urine. Haley and Bassin (11) injected rats subcutaneously with rutin, and found slightly less than 16 per cent of the rutin excreted in the urine while the values for ethereal sulfate and glucuronide were increased.

The findings of Ozawa (9) and Haley and Bassin (11) suggest that rutin and related flavonoids gaining entrance to the body fluids and tissues are metabolized to a large extent. In agreement with these findings we have had excellent circumstantial evidence for several years that oral administration of rutin or quercetin to rabbits results in appreciable absorption of these compounds from the gastrointestinal tract, and that the compounds are metabolized, the degradation products appearing in the urine in the free and conjugated forms.

Normal rabbit urine is alkaline and readily deposits a white precipitate of alkaline phosphates. When the urine was collected after administration of rutin or quercetin, the precipitate was yellow instead of white and when suspended by shaking it imparted to the whole urine sample a color closely resembling that of a solution of the chalcone of quercetin. When such a sample of urine was allowed to stand in the collecting vessel exposed to air, a darkening of the surface in contact with air took place. With passage of time the darkening became more pronounced and finally extended throughout the urine sample. Under the same conditions the color change in normal rabbit urine was negligible by comparison. When twenty-four hour samples of rabbit urine were collected before and after oral administration of quercetin and dried by lyophilization, a striking difference between the control and experimental urines was seen. The dried control urine was pale yellow while the experimental product was dark brownish-red in color.

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The visual evidence for urinary excretion of metabolic products of rutin or quercetin suggested the presence of readily oxidizable substances, such as *ortho*- or *para*-dihydroxyphenols. Chemical examination of the urine showed an increase in the excretion of phenols capable of forming indophenols when reacted with 2,6-dichloroquinonechloroimide (12). Determination of glucoronides by the method of Maughan, Evelyn, and Browne (13) showed that oral administration of quercetin resulted in the urinary excretion of at least part of the quercetin or its metabolites as glucuronides. Paper partition chromatography showed that oral administration of quercetin resulted in the urinary excretion of at least four substances not found in normal rabbit urine.

Perkin and Everest (14) have shown that the alkali fusion products of quercetin are phloroglucinol and protocatechuic acid. If the milder physiological processes degraded some of the quercetin molecules to the same end products, the presence of protocatechuic acid in the urine would provide the *ortho*-hydroxyl groups required to account for the presence of at least one easily oxidizable substance.

EXPERIMENTAL

In most of the experiments on the metabolic fate of rutin we actually administered the aglycone, which has approximately one-half the molecular weight of rutin, thus permitting maximum dosage of quercetin without increasing the total weight given. Since we were interested in obtaining maximum yield of any metabolites formed from rutin or quercetin, albino rabbits averaging 3–4 Kg. were given 2–2.5 Gm. of quercetin by stomach tube. The rabbits were placed in metabolism cages and the urine was collected in bottles containing hydrochloric acid and sodium bisulfite to retard oxidation of phenolic compounds.

Hoepfner's (15) nitrous acid reaction for phenolic compounds was applied to control and experimental urines in the following manner. Two drops of 10% acetic acid were added to two drops of urine on a spot plate, followed by the addition of two drops of 10% sodium nitrite. Two minutes later when a definite yellow color was produced, two drops of 2 *N* sodium hydroxide were added. A cherry-red color was always produced with rabbit urine after oral administration of rutin or quercetin. The same color is given by catechol, protocatechualdehyde, protocatechuic acid, caffeic acid, and chlorogenic acid, since the test is rather general for compounds having two hydroxyl groups *ortho* to each other.

The rates of reaction and the colors produced can serve as a rough means of differentiating phenolic compounds when they are reacted with 2,6-dichloroquinonechloroimide to form indophenols. This fact was utilized to differentiate the indophenol formed with one of the metabolites of quercetin from the phenol-indophenol formed with the phenol normally present in rabbit urine. When aqueous solutions of catechol and phenol are converted to indophenols as described by Tucker (16), the catechol-indophenol forms rapidly and has a characteristic reddish-purple color, whereas the phenol-indophenol forms slowly and has a blue color which cannot be confused with the color of the catechol derivative. Application of the indophenol reaction to control and to experimental urine of rabbits receiving quercetin was carried out as follows. About 5 ml. of urine were placed in a small separatory funnel, made alkaline with 1% sodium borate solution, and treated with 1 ml. of 0.05% 2,6-dichloroquinonechloroimide solution. Color developed rapidly in the experimental urines but slowly in the control samples. Before color due to phenol-indophenol developed, the content of the separatory funnel was acidified with a few drops of concentrated hydrochloric acid to stop further indophenol formation. After shaking with about 10 ml. of ether to extract the indophenol from the acidified urine and reagents, the aqueous phase was separated and discarded. When the ether extract was shaken with 5–10 ml. of sodium borate solution, the indophenol passed into the aqueous phase, giving an indophenol color characteristic of catechol as judged by visual inspection. The spectrophotometric absorption curves for catechol-indophenol and the indophenol derived from quercetin urine are quite similar but differ slightly in the region between 400 and 450 mμ. The metabolite of quercetin responsible for the characteristic indophenol was further differentiated from catechol by being strongly acidic, indicating the presence of at least one carboxyl group.

The isolation of this acidic, *ortho*-dihydroxyphenol yielding a characteristic indophenol was accomplished as follows. The twenty-four hour urine sample collected following oral administration of rutin or quercetin was extracted for a minimum of four hours with ethyl ether in a liquid-liquid extractor. The ether extract was evaporated to dryness under vacuum with the application of sufficient heat by means of a warm water bath to prevent frosting. The dry residue was re-extracted three or four times with approximately 15-ml. portions of dry ether, and the combined extracts were evaporated to near dryness under vacuum. A saturated solution of barium hydroxide was slowly added during constant agitation until the mixture was barely alkaline as shown by alk-acid test paper. A few drops of 10% acetic acid were added quickly to give a slightly acid reaction. Evaporation was carried out under vacuum until the volume was reduced to 5–10 ml. Crystallization of the barium salt, sometimes induced by scratching the container at the liquid level, took place if the liquid volume was small enough. The crystalline mass was filtered on a medium fritted glass filter and washed three or four times with 5-ml. portions of 70% alcohol. The precipitate containing the barium salt was transferred to a 200-ml. Erlenmeyer flask and made acid with 6 *N* hydrochloric acid to give a *p*H of 2. The free organic acid was extracted three or four times with 15-ml. portions of ethyl ether by vigorous agitation and decantation. The combined extracts were dried under vacuum, the residue dissolved in about 10 ml. of anhydrous ether, and placed in a crystallizing dish with 2 volumes of petroleum ether (boiling range 30–60°). Within an hour or so plate-like crystals deposited on the walls of the crystallizing dish from the turbid solution. When this did not

occur, the liquid phase was allowed to evaporate spontaneously and the residue was dried in a vacuum desiccator for at least twenty-four hours. The dry residue was then extracted with anhydrous ethyl ether and filtered through a pad of Hyflo filter aid on a medium fritted-glass filter. The filtrate was put in a crystallizing dish with 2 or 3 volumes of petroleum ether and crystallization allowed to occur. Repetition of this process of dehydration, solution in anhydrous ether, and crystallization from a mixture of ethyl ether and petroleum ether yielded crystals pure enough for identification.

RESULTS

The crystals isolated by the above procedure had an initial melting point ranging from 118 to 123°. By alternately melting and freezing the product several times the melting point became stabilized at 127°. After thorough drying of the crystals in a vacuum desiccator a melting point of 127° was always obtained.

The neutralization equivalent of the isolated crystalline compound as determined by titration with standard sodium hydroxide and phenolphthalein was 167.7. Determination of the carbon and hydrogen content gave values of 57.3% and 4.96%, respectively. The compound was strongly acidic, gave a pronounced cherry-red color by the nitrous acid reaction, and yielded an indophenol closely resembling that of catechol as seen in Fig. 1. The crystallographic characteristics were as follows.

The compound usually crystallizes from the ether solution after the addition of petroleum ether as loose clusters of colorless, micaceous plates. The plates are ragged in outline. When a few crystals are pressed under a cover glass they fracture easily. The cracks are frequently in two mutually perpendicular directions although curving cracks also occur.

In polarized light the polarization colors are of low order owing to the thinness of the plates. Extinction is parallel to the straight cleavage cracks. The plates give a centered acute bisectrix interference figure of large angle ($2E = 136°$) whose optical character is positive.

Refractive indices measured by the immersion method in sodium light at a temperature of 28° are $\alpha = 1.573$ and $\beta = 1.618$ for the ordinary view of the crystal. Lower values: $\alpha = 1.570$ and $\beta = 1.610$ have been observed on some samples, thought to be impure.

Fusion of the sample under a cover glass yields slow-growing spherulitic masses when crystallization is spontaneous but a partial melt-back of the first crystals obtained yields large micaceous plates by slow growth when the partially remelted preparation is allowed to cool slowly. The platy crystals thus obtained show shrinkage cracks to which extinction is parallel. They show the same refractive indices as the original crystals and the same interference figure.

The compound is very soluble in water from which bladed crystals may be obtained. The bladed crystals become opaque upon drying. This behavior is regarded as indicating loss of water of crystallization.

These data suggest that the compound is 3,4-dihydroxyphenylacetic acid, which has the same crystallographic characteristics, a molecular weight of 168.14 and carbon and hydrogen values of 57.15% and 4.79%, respectively.

A sample of dimethoxyphenylacetic acid was demethylated and the resulting 3,4-dihydroxyphenylacetic acid, when isolated and purified, melted at 127°. The melting point of a mixture of 3,4-dihydroxyphenylacetic acid and the compound isolated from rabbit urine was 127°. The indophenols of the synthetic 3,4-dihydroxyphenylacetic acid and the compound obtained from urine were identical as shown in Fig. 1.

A portion of the product derived from rabbit urine was methylated and its X-ray diffraction pattern was found to be identical with that of an authentic sample of dimethoxyphenylacetic acid as seen in Fig. 2.

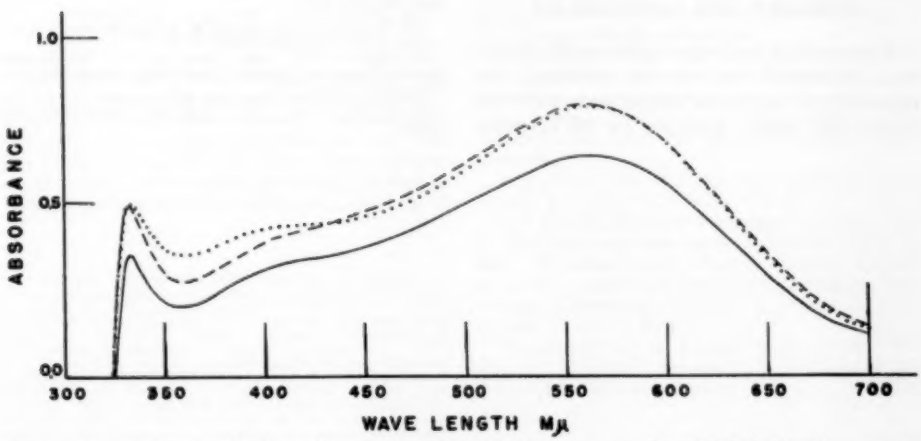

Fig. 1.—Spectrophotometric absorption curves of indophenols. ..... Catechol indophenol. - - - 3,4-Dihydroxyphenylacetic acid indophenol (synthetic). ——— Urine product indophenol.

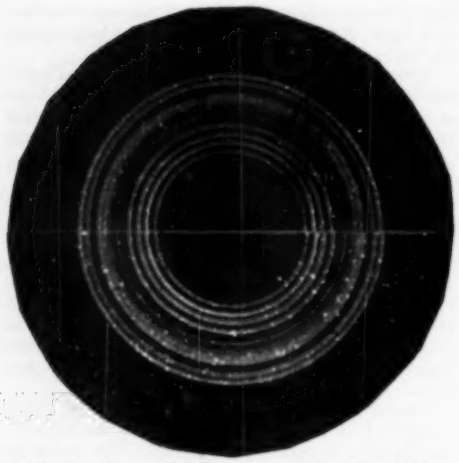

Fig. 2.—X-ray diffraction patterns. Upper half—Synthetic 3,4-dimethoxyphenylacetic acid. Lower half—Compound isolated from urine (dimethoxy derivative).

Having identified one of the metabolic products of quercetin as 3,4-dihydroxyphenylacetic acid, an experiment was designed to obtain an approximate idea of the amount of quercetin absorbed after the oral administration of 2.5 Gm. The procedure used in the isolation of the compound from urine was applied to a sample of control urine to which a known amount of 3,4-dihydroxyphenylacetic acid had been added. A recovery of 80% was obtained. The recovery of 3,4-dihydroxyphenylacetic acid from urine after a 2.5-Gm. dose of quercetin varied between 0.2 and 0.3 Gm. Assuming the same efficiency of recovery noted above and allowing for the fact that dihydroxyphenylacetic acid is about one-half the molecular weight of quercetin, the absorption of 0.6 Gm., or roughly 25% of the quercetin, was accounted for.

SUMMARY AND CONCLUSIONS

When rutin or quercetin is given orally to rabbits, substantial amounts are absorbed, metabolized, and largely excreted in the urine within twenty-four hours. Evidence for the excretion of at least four compounds has been obtained. One of these compounds has been isolated and identified as 3,4-dihydroxyphenylacetic acid, which accounts for at least 25% of the quercetin administered. Whenever a rabbit failed to void during the first twenty-four hours, the urine finally obtained gave a faintly positive test with the nitrous acid reaction, and yielded little or no crystalline product. No adequate explanation of this phenomenon is available. It may mean that longer retention in the body has permitted further degradation of the quercetin molecule.

In considering the significance of the results here reported in comparison with the contradictory reports in the literature, allowance must be made for species differences in regard to both absorption and metabolic pathways followed by absorbed flavonoids. Nevertheless, the examination of urine for the excretion of the flavonoid administered rather than its metabolic products may lead to false conclusions regarding absorption after oral administration.

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The Chemistry of *Ambrosia maritima* L.*

II. Hydrogenation, Oxidation, and Dehydrogenation of Ambrosin and Damsin

By HAMED ABU-SHADY† and TAITO O. SOINE‡

Hydrogenation, oxidation, and selenium dehydrogenation studies on ambrosin and on damsin are reported. A reaction mechanism is suggested to account for the formation of identical compounds when ambrosin and damsin are hydrogenated. Oxidation reactions indicate the presence of a terminal methylene group in both compounds. The formation of a dicarboxylic acid and application of the Blanc rule to it tends to prove that the ketone function resides in a five-membered ring.

THE ISOLATION of ambrosin ($C_{15}H_{18}O_3$) and damsin ($C_{15}H_{20}O_3$) from *Ambrosia maritima* L. and their partial characterization was reported in an earlier communication (1). These compounds were both shown to be unsaturated keto-lactones and each gave the same products, i. e., dihydroambrosin ($C_{15}H_{20}O_3$) and tetrahydroambrosin ($C_{15}H_{22}O_3$), when hydrogenated. Among other things, ultraviolet and infrared spectra strongly suggested the presence of a five-membered ring in both of these compounds. The infrared spectra also suggested the presence of a terminal methylene group.

The studies in the present paper enlarge on the probable relationships of the hydrogenation products with ambrosin and damsin and also confirm the presence of a terminal methylene group. Chemical evidence for a five-membered ring in ambrosin and damsin is also presented and leads the authors to entertain the possibility that a fused cyclopentanocycloheptane ring system might be the basic carbon skeleton for these compounds.

EXPERIMENTAL

Hydrogenation of Ambrosin and Damsin.—The hydrogenation of ambrosin was carried out using a 10% palladium-on-carbon catalyst in essentially the same way as previously described (1). The two products obtained were always dihydroambrosin ($C_{15}H_{20}O_3$) and tetrahydroambrosin ($C_{15}H_{22}O_3$). Minor variations in the procedure, e.g., quantity of solvent and catalyst, appeared to have little effect on the outcome of the hydrogenation. A microhydrogen absorption experiment[1] indicated that ambrosin took up the equivalent of 1.41 moles of hydrogen per mole of ambrosin under the above conditions.

Exactly the same result was experienced with damsin, the two products being dihydroambrosin and tetrahydroambrosin. In the microhydrogen absorption experiment, however, it was found that only the equivalent of 0.47 mole of hydrogen per mole of damsin was taken up.

Ambrosin (2 Gm.) was dissolved in methyl alcohol (75 cc.) and the solution was introduced into a Parr hydrogenation apparatus together with 0.2 Gm. of platinum oxide catalyst mixed with 5 cc. of water. An initial pressure of 40 lb. of hydrogen was used and the hydrogenation appeared to take place rather quickly although it was continued for several hours. The catalyst was removed by filtration and the solvent was then removed by evaporation. On standing overnight in a vacuum desiccator the oily residue crystallized to give 1.9 Gm. of hydrogenated material which was recrystallized once from a mixture of ether and petroleum ether (b. p. 30–60°) and three times from Skellysolve® B. The final product (0.7 Gm.) was in the form of white prisms, m. p. 85–87°. From its analytical data, chemical reactivity, and infrared spectrum (Fig. 3) it was named *dihydroambrosinol*.

Dihydroambrosinol was also obtained by the hydrogenation of dihydroambrosin under the same conditions as described above for ambrosin. The product was identified by a mixed melting point determination with the product obtained from ambrosin.

Anal.—Calc. for $C_{15}H_{22}O_3$: C, 71.97; H, 8.86. Found: C, 71.53; H, 8.76.

The acetate of dihydroambrosinol was prepared in the usual way using acetic anhydride and pyridine. It formed needle-shaped, colorless crystals, m. p. 104°, when crystallized from petroleum ether.

Anal.—Calcd. for $C_{17}H_{24}O_4$: C, 69.84; H, 8.27. Found: C, 70.01; H, 8.47.

The phenylurethan derivative, m. p. 210–212°, and the 3,5-dinitrobenzoate, m. p. 150°, were also prepared in the usual manner.

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[1] We are indebted to Dr. J. R. Kerns, analyst at the General Mills Research Laboratories, Minneapolis, Minn., for carrying out these determinations.

Oxidation Experiments.—*Nitric Acid.*—Dihydroambrosin (0.3 Gm.) was heated gently with 3 cc. of nitric acid for five minutes on a steam bath. The solution was then poured into 30 cc. of water and left overnight in the refrigerator to obtain 0.25 Gm. of slightly yellow-colored prisms, m. p. 220° (with efferv.). Repeated crystallization from boiling water yielded colorless, somewhat hygroscopic prisms, m. p. 230° (with efferv.). These crystals were soluble in most organic solvents but sparingly soluble in water. They dissolved in sodium carbonate solution accompanied by effervescence. The crystals were also soluble in fixed alkalies and could be reprecipitated by acidification with mineral acids. Efforts to prepare an oxime from this acid were without success. From a consideration of its properties it appears to be a diacid and has been provisionally named *dihydroambrosinic acid.*

Anal.—Calcd. for $C_{15}H_{20}O_6$: C, 60.83; H, 6.80; neut. equiv., 148.16. Found: C, 61.01; 61.09; H, 6.90, 7.08; neut. equiv., 150, 153.

Dihydroambrosinic acid was converted to the dimethyl ester by dissolving 0.6 Gm. of the acid in 5 cc. of methyl alcohol and then adding to this solution 30 cc. of a solution of diazomethane in ether (derived from 2.5 Gm. of N-nitrosomethylurea). The reaction was immediate and after standing overnight in an ice bath the solvent was removed by distillation. The residual crystals were recrystallized several times from dilute alcohol to give an analytical sample, m. p. 138–139° as colorless prismatic needles. The ester failed to form either an oxime or a 2,4-dinitrophenylhydrazone when treated in the usual way with the appropriate reagents. (See Fig. 3 for the infrared spectrum.)

Anal.—Calcd. for $C_{17}H_{24}O_6$: C, 62.94; H, 7.45. Found: C, 63.10, 63.09; H, 7.40, 7.36.

Dihydroambrosinic acid (2.2 Gm.) was covered with 8 cc. of acetic anhydride and the solution was then slowly distilled at atmospheric pressure. Traces of acetic anhydride were removed under reduced pressure. The temperature of the oil bath was then raised to 300° and the pressure was reduced to 3 mm. A reddish-colored oil distilled over and readily crystallized when rubbed with methyl alcohol. It was recrystallized from methyl alcohol to give 0.1 Gm. of white, needle-like crystals, m. p. 185–187°. From a consideration of the analytical data it appears that this compound is an anhydride resulting from the loss of a molecule of water from dihydroambrosinic acid.

Anal.—Calcd. for $C_{15}H_{18}O_5$: C, 64.73; H, 6.52. Found: C, 65.00; H, 6.65.

Potassium Permanganate.—Damsin (0.3 Gm.) was finely ground and introduced into a flask containing 15 cc. of 2 *N* sulfuric acid. Twenty cubic centimeters of 8% potassium permanganate solution was then added and the solution was cooled externally to maintain it at room temperature. When the oxidation was complete (0.5 hr.) the mixture was transferred to a centrifuge cup and the precipitated manganese dioxide removed by centrifugation. The supernatant liquid was removed by decantation and then filtered. The filtered liquid was then distilled to a small volume, water was added to the residue, and it was again distilled to a small volume. The distillate was acid to litmus and faintly reduced Tollen's reagent. The distillate was then neutralized with a few drops of dilute aqueous sodium hydroxide solution and then made slightly acid by the addition of one small drop of dilute hydrochloric acid. Alcohol (30 cc.) was then added together with 200 mg. of *p*-bromophenacyl bromide and the solution was refluxed for three hours. The refluxed solution was freed of alcohol by distillation under reduced pressure during which time 0.14 Gm. of solid separated. This was recrystallized from alcohol to yield slightly yellow feathery crystals, m. p. 135–136°. These crystals failed to depress the melting point of an authentic sample of *p*-bromophenacylformate, m. p. 137–139° when a mixed melting point determination was performed.

When the same oxidation was performed on ambrosin no formic acid was obtained under the above conditions.

Ozone.—A solution of damsin in 40 cc. of glacial acetic acid was ozonized for two hours with a current of oxygen containing about 3% ozone corresponding to a flow of 0.26–0.28 mM per minute. The acetic acid solution of the ozonide was diluted with an equal volume of water and steam distilled for two hours. The volume of distillate was 700 cc. Treatment of 100 cc. of the distillate with 150 cc. of a saturated aqueous solution of dimedone resulted in the deposition of 100 mg. (in 24 hr.) of the dimedone derivative of formaldehyde, m. p. 188–189° The dimedone derivative was identified by a mixed melting point determination with an authentic sample. The amount of dimedone derivative represented 33% of the theoretical amount of formaldehyde obtainable from one terminal methylene group. Another 100-cc. portion was treated with a saturated alcoholic solution of 2,4-dinitrophenylhydrazine and the solution was then concentrated to a small volume. On cooling, yellow crystals of the 2,4-dinitrophenylhydrazone of formaldehyde separated, m. p. 159–161°, identified by a mixed melting point with an authentic sample.

The residue left after the steam distillation was transferred to a separatory funnel and shaken repeatedly with small volumes of ether. The ethereal extracts were combined, washed once with water, and dried over anhydrous sodium sulfate. The ether was removed by evaporation and the residue was treated with 5% aqueous sodium carbonate solution and filtered. The filtrate was acidified with hydrochloric acid and an oily material separated but failed to crystallize. It resisted all attempts at crystallization. The oily material gave a powerful iodoform test and reacted with 2,4-dinitrophenylhydrazine to give a very small amount of the hydrazone. However, the amount of product was too small to identify further.

Ambrosin was subjected to exactly the same treatment as that described for damsin and likewise yielded formaldehyde in the distillate to the extent of 35.4% of the theoretical amount obtainable from one terminal methylene group. The residues from the steam distillation also yielded an uncrystallizable oil giving a powerful iodoform test and exhibiting definite acidic properties.

Dehydrogenation Experiment.—Ambrosin (16 Gm.) was hydrogenated as completely as possible with platinum oxide catalyst in methyl alcohol using the conventional Parr catalytic hydrogenation apparatus. The solvent was removed under reduced pressure and the residue mixed with 16

Gm. of selenium without further purification. The mixture was then heated continuously at 300–310° for forty-three hours. The dark-brown residue was extracted with 800 cc. of ether and the ethereal extract was filtered and dried. Removal of the ether on a steam bath left a dark-brown residue that was distilled *in vacuo*. Two principal fractions were collected, namely, a slightly yellow-colored one, m. p. 120–130° (12 mm.) and a blue-colored fraction over a range of 130–175° (8 mm.). The latter fraction became more intensely blue and more viscid as the distillation proceeded. The amounts obtained in each case, however, were quite small and it was further noted that when the blue distillate was permitted to stand overnight there was a noticeable change in color toward purple. The blue-colored distillate was dissolved in petroleum ether, cooled, and extracted with 85% phosphoric acid after the manner of Plattner and Pfau (2) in handling azulenes. The acid extract was covered with ether, ice-cold water added, and the whole extracted thoroughly with ether. The blue-colored ethereal extracts were combined, dried, concentrated, and treated with a saturated alcoholic solution of trinitrobenzene. On standing in the refrigerator the solution deposited a few milligrams of dark violet-colored needles. These were crystallized once from alcohol to give a sample, m. p. 125–130°. Further purification was impossible because of the small amount of derivative. An analysis on these admittedly impure crystals gave the following results:

Anal.—C, 58.94; H, 5.05; N, 11.86.

The slightly yellow-colored fraction was converted to the trinitrobenzene derivative in the usual manner. The long yellow needles were recrystallized from alcohol four times to give an analytical sample, m. p. 151–153° with previous softening.

Anal.—Calcd. for $C_{19}H_{17}N_3O_8$: C, 59.53; H, 4.47; N, 10.99. Calcd. for $C_{19}H_{19}N_3O_8$: C, 59.21; H, 4.97; N, 10.90. Found: C, 59.29; H, 4.47; N, 11.29.

DISCUSSION

The hydrogenation of ambrosin and damsin had been shown earlier to proceed to the same products, i. e., dihydroambrosin (isomeric with damsin) and tetrahydroambrosin when a palladium-on-carbon catalyst was used. In each case, all of the ambrosin or damsin could be accounted for as dihydroambrosin or tetrahydroambrosin and it should be emphasized that each hydrogenation product had double the C-methyl content of the original ambrosin or damsin. In the present work, microhydrogen absorption experiments have shown that, whereas ambrosin takes up hydrogen equivalent to 1.41 double bonds, damsin takes up only the equivalent of 0.47 double bond. With respect to damsin, therefore, it becomes evident that two competing reactions must be assumed to account for the formation of these products: (*a*) *addition* of hydrogen to a terminal methylene group to account for the tetrahydroambrosin and (*b*) a *rearrangement* with *no* hydrogen pickup to account for the dihydroambrosin. That the same occurs in ambrosin during hydrogenation is an attractive hypothesis, particularly in view of the close relationship between the two compounds as demonstrated by identical hydrogenation products. However, it must be pointed out that ambrosin has not yet been converted to damsin by hydrogenation. Tentatively, it can be assumed that the conjugated linkage in ambrosin probably accounts for the absorption of one full mole of hydrogen and that, during the absorption of hydrogen, the same rearrangement mechanism as outlined for the conversion of damsin to dihydroambrosin is in effect. This would account for the formation of both hydrogenation products from ambrosin. The scheme depicted in Figure 1 suggests an interrelationship between ambrosin, damsin, and their hydrogenation products. Of course, an objection to this scheme is the usually greater difficulty of adding hydrogen to conjugated systems than to isolated double bonds.

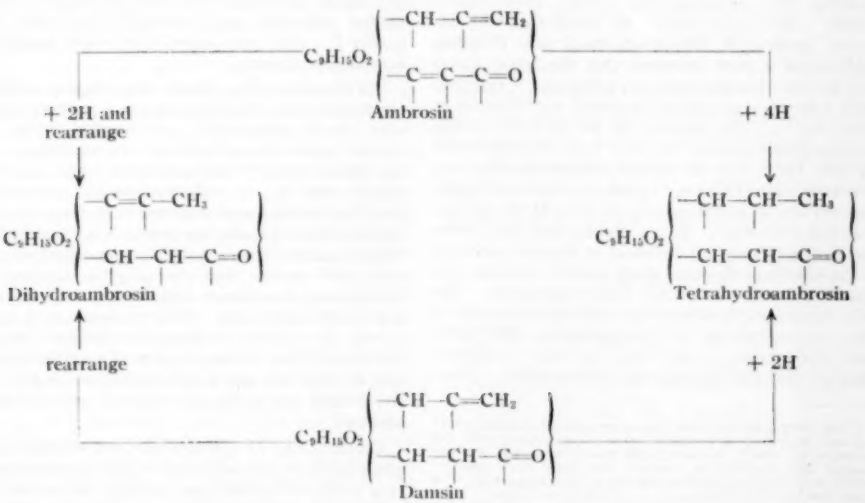

Figure 1.

However, it is well known that highly hindered isolated double bonds, as for example in $\Delta^{8(14)}$-cholestenol, are completely stable to hydrogenation under comparable conditions (3). In accordance with the experimental evidence presented in this paper it is entirely possible that the hydrogenation resistant unconjugated double bond in dihydroambrosin could be at one of the bridge heads of a fused cyclopentanocycloheptane ring system. This would be entirely analogous to the situation in $\Delta^{8(14)}$-cholestenol and would account for its resistance to hydrogenation. It must be emphasized, however, that with the evidence in hand the presumption of a seven-membered ring in ambrosin and damsin is highly speculative and based only on the formation of a blue azulenic[3] compound on dehydrogenation with selenium. That a five-membered ring exists in these two compounds appears very probable, however, on the basis of the evidence to be discussed later in this paper. The ready isomerization of a double bond into a highly hindered position under similar conditions of catalytic hydrogenation is not unprecedented. It may be compared favorably with the isomerizations (Fig. 2) of 7-dehydrocholesterol and Δ^7-cholestenol wherein a $\Delta^{7,8}$-double bond isomerizes into the $\Delta^{8,14}$-position under similar conditions.

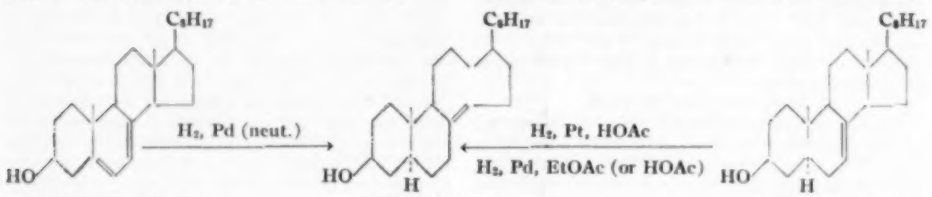

Figure 2.

It has not yet been possible to obtain tetrahydroambrosin from dihydroambrosin by further hydrogenation because when platinum oxide is used as a catalyst the carbonyl group undergoes hydrogenation yielding the corresponding alcohol provisionally named *dihydroambrosinol*. In comparing the infrared spectra of dihydroambrosin and dihydroambrosinol it may be noted that the latter has a less intense carbonyl band (at 1,764 cm.$^{-1}$) together with a much more intense hydroxyl contribution at 3,597 cm.$^{-1}$. The presence of the hydroxyl group in dihydroambrosinol has also been demonstrated by the formation of typical alcohol derivatives. However, the 1,672 cm.$^{-1}$ peak, ascribed to unconjugated C=C, is still present in spite of the hydrogenation procedure. It is a curious fact that, when the hydrogenation of ambrosin or damsin proceeds to the dihydroambrosinol stage, most of the material can be accounted for as this single compound. The only logical explanation that can be advanced is that, unaccountably, the hydrogenation with platinum oxide takes a route more or less exclusively through the dihydroambrosin intermediate. However, it must also be noted that occasionally hydrogenations with this catalyst stop at the dihydroambrosin-tetrahydroambrosin stage and proceed no further. These deviations from the regular pattern of hydrogenation have been noted only with certain batches of catalyst and not with others. Experiments are in progress to determine still further the relationships of the various products of hydrogenation.

Mild nitric acid oxidation of dihydroambrosin yields a dicarboxylic acid which has been named *dihydroambrosinic acid*. This acid retains all of the carbon atoms of the original ambrosin and readily forms a dimethyl ester when treated with diazomethane. The dimethyl ester as well as the acid failed to form an oxime or 2,4-dinitrophenylhydrazone strongly suggesting that the oxidation involved the carbonyl group. It was not possible to discern the loss of the carbonyl group from the infrared spectrum because of the obscuring effect of the carbonyls in the lactone group and the two newly created carboxyl groups. It is interesting to note that the absorption at 1,672 cm.$^{-1}$ (ascribed to unconjugated C=C) is still present in dihydroambrosinic acid just as it was in dihydroambrosinol, an indication of its peculiar stability. Incidentally, the formation of a dicarboxylic acid rather than a keto-acid in this oxidation rules out the possibility that the ketone carbonyl might have substituents on both α-carbon atoms. However, the possibility of a single substituent α to the carbonyl is not excluded although some indication had been noted earlier (1) that both α-positions might possibly be free of substituents.

To determine the size of the original carbonyl-containing ring, dihydroambrosinic acid was heated with acetic anhydride and distilled. This procedure yielded an anhydride. In accordance with the Blanc rule (4) this treatment when applied to diacids with six- or seven-membered carbon chains yields cycloalkanones whereas, with four- and five-carbon chain diacids, the product is an anhydride. The formation of an anhydride from dihydroambrosinic acid implies that the original carbonyl-containing ring in ambrosin and damsin was very likely a five-membered ring. This confirms, to a certain extent, the inferences drawn in the first paper of this series (1) as to the presence of a cyclopentenone ring in ambrosin and a cyclopentanone ring in damsin (based primarily on infrared and ultraviolet spectra).

Confirmation of the presence of a terminal methylene group in ambrosin and damsin was obtained by two oxidative procedures, namely, potassium permanganate oxidation and ozonolysis. In the case of damsin the permanganate oxidation mixture,

[3] The presumption that this blue-colored compound is azulenic in nature is certainly open to argument with the evidence at hand. However, the mere fact that a blue-colored dehydrogenation product has been obtained, together with its hydrocarbon nature, is very suggestive of a highly conjugated system such as is found in azulenes. The demonstration, both spectrophotometrically and chemically, of the presence of a five-membered ring in the parent compounds lends further credence to the above suggestion.

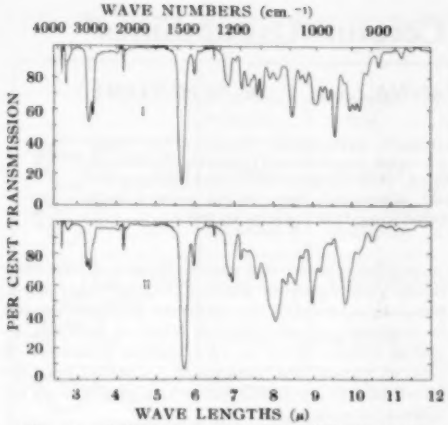

Fig. 3.—Infrared spectra. I. Dihydroambrosinol; II. Dimethyl ester of dihydroambrosinic acid.

when distilled, revealed the presence of formic acid in the distillate. Ambrosin, for some unexplained reason, failed to yield formic acid on similar treatment. However, ozonolysis of both ambrosin and damsin, followed by distillation, yielded formaldehyde (30–35% of theory) in the distillate. The formation of significant amounts of formic acid in one case and formaldehyde in the other confirms the suspected presence of a terminal methylene group. Unfortunately, no crystalline product could be isolated from the nonvolatile residues from the ozonolysis of either damsin or ambrosin. In each case, however, the residue gave a good iodoform test and exhibited an acid character by effervescing carbonates.

To further ascertain the basic ring structure of these natural products they were hydrogenated as completely as possible and then subjected to a selenium dehydrogenation. Two principal products were obtained by this procedure, namely, an intensely blue-colored oil and a slightly yellow-colored oil.

The blue-colored oil is being considered tentatively as an azulene on the basis of its color, its hydrocarbon nature, and the demonstrated presence of a five-membered ring in the original lactones. It is not surprising to obtain such a small yield of azulene when one considers the highly oxygenated character of the original compounds. It has been demonstrated by others that azulenes can be obtained by dehydrogenation from compounds not originally containing a five-membered ring as in the case of elemol (5) and ledol (6). Nevertheless, the fact that a five-membered ring is present in ambrosin and damsin lends strong support to the assumption that an azulene has, indeed, been formed.

The slightly yellow-colored oil analyzes for the formula, $C_{13}H_{14}$, but does not appear to be azulenogenic in character as evidenced by a negative reaction to the test of Sabetay (7). Whether or not this compound is naphthalenic in character must await preparation of larger quantities of the material and further studies into the dehydrogenation of the original compounds. Certainly, with the facts in hand, it does not appear that the compound could be azulenic and it is entirely possible that rearrangements could take place under the conditions of the dehydrogenation to yield a naphthalenic residue. The loss of two carbon atoms during the selenium dehydrogenation of a sesquiterpene is not unusual and has been recorded in the literature during the dehydrogenation of various similar compounds (8–10). Almost invariably, one of the carbons that is lost during a selenium dehydrogenation is a tertiary methyl group together with opening of the lactone ring and loss of another carbon at that point. Whether or not this is so in ambrosin and damsin is a matter for speculation but it does provide a strong reason for suspecting the presence of a tertiary methyl group in these compounds.

Further dehydrogenations are being carried out to obtain larger amounts of these materials in order to throw more light on their structures.

SUMMARY

Hydrogenation experiments on ambrosin and damsin suggest a mechanism to account for the formation of identical hydrogenation products from both compounds. Similar reactions by certain sterol compounds lend support to the suggested mechanisms.

Oxidation studies have pointed definitely to the presence of a terminal methylene group in both ambrosin and damsin. Nitric acid oxidation has provided a dicarboxylic acid by means of which the Blanc rule has been applied to prove that the ketone function is present in a five-membered ring. The formation of a dicarboxylic acid also implies that at least one position adjacent to the ketone carbonyl is free of substituents and confirms an earlier conclusion to this effect based on the formation of a monopiperonylidene derivative of ambrosin.

Selenium dehydrogenation has yielded an intensely blue-colored hydrocarbon that is very likely azulenic in nature. An accompanying unidentified, slightly yellow-colored hydrocarbon has been obtained with the formula, $C_{13}H_{14}$. On the basis of the behavior of similar lactones during selenium dehydrogenation it is very probable that the loss of at least one of the carbons is due to its character as a tertiary methyl group.

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Fungistatic Activity of Certain Azomethines*

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Thirteen azomethines were tested against several pathogenic fungi. The fungistatic activity of the aldehyde constituent was more pronounced than the other components of the azomethines. The azomethine (3,4-methylenedioxybenzylidene-4-carboxyaniline) and the component aldehyde, piperonal, were found to have high *in vitro* fungistatic potency while exhibiting low toxicity in mice.

INITIAL EVALUATION of potential fungicides is usually based on the concentration of the test compound needed for complete inhibition of fungal growth over a short period of time *in vitro*. In a study designed to evaluate the fungistatic activity of some azomethines it was found desirable to test a number of concentrations of these azomethines to obtain inhibited fungal growth at the fifty per cent point. This report is concerned with the testing of some azomethines related to *para*-aminobenzoic acid in an attempt to learn about the structural requirements of these compounds for fungistatic activity. This study was carried out on the following fungi: *Trichophyton mentagrophytes, Blastomyces dermatitidis, Histoplasma capsulatum, Candida albicans, Sporotrichum Schenkii,* and *Aspergillus amstelodami.*

EXPERIMENTAL

Known amounts of the azomethines and related compounds were dissolved in water by adding a small amount of ethanol or sodium bicarbonate and warming. The solution was mixed with partially cooled Sabouraud's agar (*p*H 6.8 after autoclaving) under sterile conditions and poured into Petri dishes, allowed to harden, and then inoculated with the test organisms. The test organisms were grown at room temperature for sixteen days. The relative growth was computed by measuring the colony diameters in centimeters from each of the three plates and then calculating the average diameter. In general, colony diameters were found to be quite similar on each set of plates. Triplicate plates were used for each concentration of the test compound. A sufficient number of concentrations of the test compound (azomethine) were run simultaneously with control plates to determine the concentration of azomethine needed to inhibit the growth of the test plates 50% and 100% of that of the control plates.

In some cases the toxicity of the azomethine and its aldehyde portion were determined in mice. For this purpose the azomethines, which are, in general, quite insoluble in common injection media, were suspended in water and administered orally to white mice. The aldehyde portion, on the other hand, being lipoid-soluble was injected intraperitoneally in propylene glycol. In this latter, a constant injection volume of 0.1 cc. of propylene glycol per 30 Gm. of mouse was maintained. Control mice injected at this level with propylene glycol alone exhibited no toxic signs.

RESULTS AND DISCUSSION

The data for the concentration of the various compounds tested to produce a 50% inhibition of growth in sixteen days on Sabouraud's agar are summarized in Table I. It was found that a shorter period of time could have been used to calculate the 50% inhibition point, but a test period of ten days or shorter gave erratic results especially with the slower growing pathogens. It may be seen from Table I that certain azomethines are quite effective against some of the pathogens, which might suggest possible clinical usefulness.

Of greatest interest was the azomethine 3,4-methylenedioxybenzylidene-4-carboxyaniline which exhibited, on the whole, the highest over-all activity in the series (see Fig. 1). In an attempt to deter-

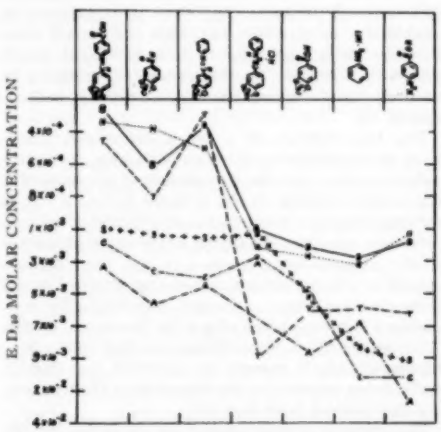

Fig. 1.—Molar concentration of the azomethine, 3,4 - methylenedioxybenzylidene - 4 - carboxyaniline, and related compounds necessary to inhibit the *in vitro* growth of *B* = *B. dermatitidis*, *H* = *H. capsulatum*, *T* = *T. mentagrophytes*, *S* = *S. Schenkii*, *C* = *C. albicans*, and *A* = *A. amstelodami*. This figure was drawn from Table I to show the relationship between the parent azomethine and its possible degradation products.

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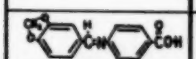
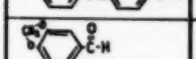
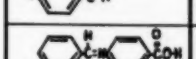
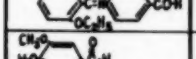
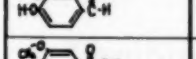
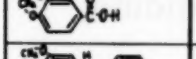
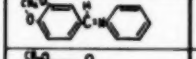
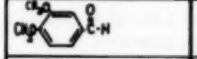
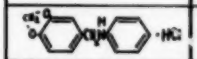
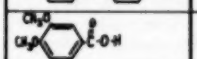
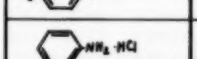
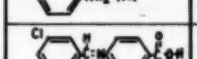
TABLE I.—MOLAR CONCENTRATION OF AZOMETHINES AND RELATED COMPOUNDS NECESSARY TO INHIBIT THE IN VITRO GROWTH OF THE TEST FUNGI FIFTY PER CENT

	B. DERMATITIDIS	H. CAPSULATUM	T. MENTAGROPHYTES	S. SCHENKII	C. ALBICANS	A. AMSTELODAMI
	0.00026	0.00041	0.00045	0.0011	0.0018	0.0033
	0.00061	0.00036	0.00078	0.0013	0.0035	0.0052
	0.0011	0.00052	0.00071	0.0014	0.0034	0.0018
	0.0016	0.0019	0.0032	0.0020	0.0011	0.0084
	0.0022	0.0027	0.0059	0.0056	0.0047	0.0088
	0.00031	0.00051	0.00029	0.0018	0.0041	0.0043
	0.00017	0.0018	0.0039	0.0060	0.0048	0.0090
	0.0014	0.0018	0.0091	0.0015	0.0027	0.0029
	0.0016	0.0011	0.0039	0.0044	0.0046	0.0093
	0.0029	0.0031	0.0058	0.0083	0.011	0.0067
	0.00009	0.00004	0.00018	0.00037	0.0022	0.0025
	0.00083	0.00088	0.0012	0.0015	0.0033	0.0054
	0.0018	0.0016	0.0061	0.0093	0.012	0.028

mine more about the properties of 3,4-methylenedioxybenzylidene-4-carboxyaniline essential for this fungistatic activity, the component parts and possible metabolized products were prepared and studied against the test fungi (Fig. 1). It should be noted that against *B. dermatitidis*, *H. capsulatum*, and *T. mentagrophytes* the aldehyde component (piperonal) of the azomethine on a molar basis is equal in fungistatic activity to the azomethines when the fungistatic activity of the amino component of the azomethine is added to that of the aldehyde. The other possible degradation or metabolic products of the azomethines such as *para*-aminobenzoic acid, piperonylic acid, *n*-phenyl-3,4-methylenedioxybenzylamine and aniline were much less active against the above three pathogens on a molar basis. This might suggest that the azomethines are broken down slowly by the above pathogens, liberating the aldehyde and the amino components of the azomethines. Thus one might conjecture the azomethines serve as a precursor of the fungistatic aldehydes. Such, however, is not the situation with *S. Schenkii*, *C. albicans*, and *A. amstelodami* since this pattern was not exhibited.

As to the over-all fungistatic activity of the azomethines against the fungi studied, it is indicated in Fig. 1 that the fungi *B. dermatitidis*, *H. capsulatum*, and *T. mentagrophytes* are about equal in sensitivity to the various azomethines and related compounds while these compounds affect *S. Schenkii*, *A. amstelodami*, and *C. albicans* to a lesser extent.

Toxicity studies were carried out only with the most promising substance in the series, that is, 3,4-methylenedioxybenzylidene-4-carboxyaniline. This azomethine possessed an LD_{50} above 500 mg./Kg. when administered orally in water suspension to mice. The LD_{50} of its component aldehyde, administered orally in water suspension, is also above 500 mg./Kg. When the aldehyde was injected in propylene glycol solution, the LD_{50} by the intraperitoneal route was 480 mg./Kg. Mice died of respiratory failure following a depressed phase. Upon opening the chest cavity the heart was still beating.

SUMMARY

1. Thirteen organic compounds were screened against *Trichophyton mentagrophytes*, *Blastomyces dermatitidis*, *Histoplasma capsulatum*, *Candida*

albicans, *Sporotrichum Schenkii*, and *Aspergillus amstelodami*.

2. The aldehyde constituent was found to be more toxic to the test organisms than the other components of the azomethines.

3. The azomethine (3,4-methylenedioxybenzylidene-4-carboxyaniline) and the component aldehyde, piperonal, were found to have high *in vitro* fungistatic activity while exhibiting low toxicity in mice.

The Anesthetic Activity of Some Amidines*

By OLE GISVOLD†

The anesthetic activity of some esters of *p*-carboxybenzamidine and N-alkyl *p*-carboxybenzamidine have been reported. Mention is also made of their bactericidal and antispasmodic activities.

THE PREPARATION and anesthetic activity of a number of esters of *p*-carboxybenzamidine have been reported (1). Where the alcohol portion of the molecule was represented by the primary alcohols with unbranched stem nuclei of methyl through hexyl a maximum activity (145 min. for a 1 per cent solution administered intracutaneously in guinea-pig skin) was obtained with the hexyl derivative. Other alcohols were benzyl alcohol, cyclohexanol, and several simple halogenated alcohols. Some of these compounds (see Table I) were considerably more active than cocaine when instilled in guinea-pig eyes and procaine when administered intracutaneously in guinea-pig skin. Toxicities were favorable; however, they produced an undesirable pruritis when injected in the forearm of man. Because the above leads showed some promise, several new esters of *p*-carboxybenzamidine and the N-alkyl analogs thereof were prepared in order to expand our knowledge in this field. The preparation of these compounds has previously been reported in THIS JOURNAL (2).

EXPERIMENTAL

This is a progress report in this field. Tests for the anesthetic activity of these compounds were conducted by C. L. Rose of Eli Lilly and Company. The results of these assays are tabulated in Table I.

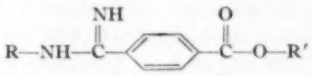
It is regrettable that sufficient amounts of a number of the above compounds were not prepared so that their partition coefficients and their *p*Ka values could be determined. It is well known that increased liposolubility within certain limits in a given series of compounds is usually associated with an increase in anesthetic activity. Often minor changes in *p*Ka values effect significant changes in biological activity. For example, Doebner's Violet has a *p*Ka of 5.38 and is 2% ionized at a *p*H of 7.3. At this *p*H it is bacteriostatic in 1:20,000 concentration for *Staphylococcus aureus*. Malachite Green on the other hand has a *p*Ka of 6.9 and is 28% ionized at a *p*H of 7.3. At this *p*H it is bacteriostatic in 1:80,000 concentration for *S. aureus* (3). However in the series of amidines prepared, the differences in their *p*Ka values is expected to be of a low order. The differences in their partition coefficients may be the chief factor in determining the variations of their anesthetic activities.

Table I shows that the unsubstituted amidines are generally more active than the corresponding lower molecular weight N-alkyl derivatives. However, as the size of the N-alkyl group is increased, the activity of the unsubstituted amidine is restored or exceeded. These differences in activity may be a function of their partition coefficients or their *p*Ka values or both.

Most of the amidines were bactericidal in concentrations of 1:1,000 against *S. aureus* and *Salmonella typhosa*. The hexahydrobenzyl ester of *p*-carboxybenzamidine, for example, was bacteriostatic at 1:10,000 but not at 1:20,000. In the bactericidal tests, 0.5-ml. aliquots of the test cultures were exposed to 5-ml. quantities of appropriate aqueous dilutions of the drug for five minutes at 20°, subcultured in tubes of beef broth and incubated at 37° for seven days. The tubes were examined daily for visible evidence of growth. For the bacteriostatic tests, drug dilutions were made in beef broth extract, and each tube inoculated with a loopful of test culture. As in the case of the germicidal tests, the tubes were incubated at 37° for seven days. These tests were performed by H. M. Powell of Eli Lilly and Company.

* Received August 21, 1953, from the University of Minnesota, College of Pharmacy, Minneapolis 14, Minn.
Presented to the Scientific Section, A. PH. A., Salt Lake City meeting, August, 1953.
† Professor of Pharmaceutical Chemistry, College of Pharmacy, University of Minnesota.

TABLE I.—ANESTHETIC ACTIVITY OF SOME ESTERS OF *p*-CARBOXYBENZAMIDINE



Compound R=	Compound R'=	Conc. of Solution, %	Duration of Anesthesia, Guinea Pigs—Eyes, Min.	Duration of Anesthesia, Guinea Pigs—Skin, Min.
H	2-Chloroethyl[a]	1	0	155[c]
Methyl	2-Chloroethyl	1	0	30
n-Propyl	2-Chloroethyl	1	0	40
n-Butyl	2-Chloroethyl	1	8–10[d]	60
H	Benzyl[e]	1	108[a]	180
Methyl	Benzyl	1	90[b]	180[a]
Ethyl	Benzyl	1	60	300[b]
n-Propyl	Benzyl	1	80	360[b]
n-Butyl	Benzyl	1	300	420+[a]
H	Hexahydrobenzyl	1	390	420+[a]
Ethyl	Hexahydrobenzyl	1	90[a]	160[a]
H	Cyclohexyl[e]	1	130[b]	360
Ethyl	Cyclohexyl	1	150	360[a]
n-Propyl	Cyclohexyl	1	160+[b]	160+[b]
n-Butyl	Cyclohexyl	1	375+[b]	375+[b]
H	Cyclopentyl	1	120[a]	280
Ethyl	Cyclopentyl	1	40	45

[a] Irritating; [b] very irritating; [c] irritation in rabbits' skin negligible; [d] onset 6 min.; [e] the anesthetic activity of these compounds have been previously reported but have been included here for convenience of comparison.

Bactericidal and bacteriostatic activity might be a very desirable feature of an anesthetic for compounding purposes and for use as surface anesthetics.

The antispasmodic activity of all of these compounds is very weak.

REFERENCES

(1) DiGangi, F. E., and Gisvold, O., THIS JOURNAL, **38**, 154(1949); Chow, A. W., and Gisvold, O., *ibid.*, **41**, 202 (1952).

(2) Rost, W., and Gisvold, O., *ibid.*, **42**, 371(1953).

(3) Albert, A., "Selective Toxicity with Special Reference to Chemotherapy," John Wiley & Sons, Inc., New York, 1951, p. 40.

Composition of Gum Turpentines of Pines*

XIX. A Report on *Pinus ponderosa* from Arizona, Colorado, South Dakota, and Northern Idaho

By P. M. ILOFF, Jr., and N. T. MIROV

The composition of gum turpentines of Ponderosa pine from four widely separated localities has been analyzed. Of interest is the presence of small amounts (1–2 per cent) of methyl chavicol in three varieties of Ponderosa pine, a large percentage of Δ^3-carene and about 5 per cent of β-myrcene in the turpentine of a variety from northern Idaho.

Pinus ponderosa is essentially a Western American species. It occupies an enormous patchy area from southern California to British Columbia and eastward to Arizona, Colorado, western Nebraska and the Black Hills of South Dakota. It is conventionally subdivided into the Pacific Coast form and the Rocky Mountain form, the latter being called variety *scopulorum*. In 1913 Schorger (7) reported that the turpentine obtained from the Pacific Coast form in California was levorotatory and contained about 5 per cent *l*-α-pinene, 60–70 per cent *l*-β-pinene and 20–25 per cent *l*-limonene. The *scopulorum* variety from Arizona, according to Schorger, was dextrorotatory and possessed turpentine of a just reversed chemical composition: 60–70 per cent *d*-α-pinene, about 5 per cent *l*-β-pinene and 20–25 per cent *l*-limonene. As Δ^3-carene was discovered by Simonsen in 1920, this terpene was not reported by Schorger as an ingredient of ponderosa pine turpentine. Later analysis of Ponderosa pine turpentine from the west slope of the Sierra Nevada of California, made by Haagen-Smit and his collaborators (5), showed the fol-

* Received September 28, 1953, from the California Forest and Range Experiment Station, maintained by the Forest Service, U. S. Department of Agriculture, in cooperation with the University of California, Berkeley.

The work reported in this paper was aided through a grant from the Rockefeller Foundation.

lowing composition: approximately 51 per cent *dl*- and *l*-β-pinene, 20 per cent *dl*- and *l*-Δ^3-carene, 25 per cent *l*-limonene and dipentene, 3 per cent *dl*- and *d*-cadinene and less than 1 per cent of a sesquiterpene possessing two double bonds and two rings, one of which is labile; its trihydrochloride had a melting point of 129–130°. No α-pinene was detected.

Ponderosa pine turpentine from southwestern Utah, investigated by Mirov (6), contained: 45 per cent *dl*- and *d*-α-pinene, 40 per cent Δ^3-carene, 2 or 3 per cent *l*-limonene and 10 per cent longifolene.

Goldblatt (4) analyzed turpentine obtained by acetone extraction of Ponderosa pine stumpwood from Oregon. The composition was: Δ^3-carene 70%; α-pinene 8 per cent; β-pinene 6 per cent; β-myrcene 8 per cent; and smaller percentages of camphene, α-terpinene, limonene, terpinolene, and *p*-cymene.

This paper deals with the results of analysis of Ponderosa pine turpentines collected at four localities of the range of this species: McNary, Ariz.; Manitou, Colo.; Black Hills of South Dakota; and Priest River, northern Idaho.[1]

The oleoresin samples were distilled *in vacuo*, so that at the end of distillation the pot temperature was 180° and the pressure was 0.1 mm.

The physical properties of the fractions from the distillations described below are shown in Table I.

[1] Thanks are due to Bruce Elmore and A. C. Hunt of the Southwest Lumber Mills, Inc., McNary, Ariz.; to District Ranger James Sykes of Pactola, S. Dak.; Austin E. Helmers, Priest River Experiment Forest, for their generous assistance in collecting the oleoresin samples.

TABLE I.—FRACTIONAL DISTILLATION OF TURPENTINES OF P. PONDEROSA FROM BLACK HILLS; P. PONDEROSA FROM PRIEST RIVER, IDAHO; P. PONDEROSA FROM MANITOU, COLO., AND P. PONDEROSA FROM MCNARY, ARIZ.

Fractions	Pressure, mm.	Boiling Range, °C.	Distillate, %	Density	Index of Refraction	Specific Rotation
				d_4^{25}	n_D^{25}	$[\alpha]_D^{25}$
Pinus ponderosa from Black Hills, S. Dak. (987 Gm. Used)						
1	760	165–167	27.0	0.8537	1.4705	−2.1
2	760	167–169	25.8	0.8549	1.4719	−1.5
3	760	169–172	19.6	0.8561	1.4714	−1.0
4	9	52–57	3.0	0.8574	1.4718	−3.1
5	9	57–64	1.3	0.8510	1.4746	−17.1
6	9	64–67	0.4	0.8514	1.4820	−14.9
7	9	67–72	1.1	0.8540	1.4819	−7.0
8	9	72–92	0.6	0.8937	1.4890	−0.7
9	9	92–96	0.7	0.9479	1.5011	+2.9
10	9	96–106	0.4	0.9442	1.4932	+9.9
11	9	106–120	4.1	0.9303	1.4959	+36.5
12	8	113–115	7.8	0.9287	1.5017	+43.7
13	3	101–117	1.1	0.9180	1.5038	+37.4
14	3	117–140	0.3	0.9224	1.5047	+33.6
15	1.5	135–150	0.5	0.9120	1.5037	+18.6
Pot residue			3.6			
Losses			2.7			
Pinus ponderosa from Priest River, Idaho (733 Gm. Used)						
				d_4^{25}	$n_D^{29.5}$	$[\alpha]_D^{25}$
1	16	51–54	2.5	0.8534	1.4698	−28.2
2	16	54–55	4.1	0.8546	1.4733	−23.7
3	16	55–57	4.1	0.8547	1.4747	−18.9
4	16	57–60	3.3	0.8519	1.4739	−8.7
5	16	60–61	13.2	0.8484	1.4738	+1.7
6	15	60–61	19.4	0.8460	1.4730	+8.9
7	15	61–63	33.3	0.8530	1.4726	+13.3
8	15	63–66	4.2	0.8504	1.4742	−21.4
9	15	66–69	1.2	0.8441	1.4762	−69.2
10	15	69–73	1.0	0.8458	1.4795	−59.6
11	15	73–78	2.6	0.8542	1.4851	−11.4
12	15	78–85	0.8	0.8755	1.4829	−11.0
13	15	85–90	0.4	0.8868	1.4821	−21.4
14	15	90–95	0.6	0.9107	1.4877	−17.3
15	15	95–106	2.0	0.9423	1.5020	−0.9
16	15	106–116	1.2	0.9514	1.5018	+18.4
17	15	116–125	1.3	0.9489	1.4940	−6.1
18	12	123–125	0.9	0.9243	1.5057	−26.0
19	2	to 133	0.9	0.9497	....	....
Pot residue and loss			3.0			

TABLE I.—CONTINUED

Fractions	Pressure mm.	Boiling Range, °C.	Distillate, %	Density	Index of Refraction	Specific Rotation
		Pinus ponderosa from Manitou, Colo. (936 Gm. Used)				
				d_4^{24}	n_D^{23}	$[\alpha]_D^{24}$
1	758	158–164	1.4	0.8567	1.4683	−0.3
2	758	164–166	1.8	0.8545	1.4709	−0.3
3	758	166–168	24.8	0.8544	1.4718	+0.3
4	758	168–171	41.4	0.8570	1.4722	+3.6
5	758	171–173	7.7	0.8588	1.4724	+1.0
6	10	55–63	2.6	0.8563	1.4739	−9.0
7	10	63–65	1.4	0.8525	1.4800	−14.2
8	10	65–67	0.8	0.8540	1.4833	−4.4
9	10	67–82	0.5	0.8646	1.4832	−2.7
10	10	82–86	0.3	0.9289	1.4938	....
11	10	86–96	1.0	0.9444	1.4972	−2.4
12	10	96–103	1.8	0.9397	1.4942	+26.1
13	10	103–106	6.7	0.9313	1.5008	+41.0
14	1	75–78	0.8	0.9202	1.5030	+32.6
15	1	Above 78	0.4	0.9169	1.5043	+55.5
Pot residue and loss			6.6			
		Pinus ponderosa from McNary, Ariz. (682 Gm. Used)				
				d_4^{23}	n_D^{23}	$[\alpha]_D^{23}$
1	14	44–47	17.5	0.8547	1.4649	+33.1
2	14	47–48	5.8	0.8541	1.4654	+29.9
3	15	48–50	5.0	0.8566	1.4666	+24.4
4	15	50–53	5.6	0.8616	1.4704	+11.2
5	15	53–55	3.9	0.8603	1.4727	−2.1
6	15	55–58	5.7	0.8597	1.4741	−7.6
7	15	58–60	4.9	0.8602	1.4738	−2.9
8	15	60–61	14.6	0.8605	1.4721	+10.3
9	15	61–63	12.1	0.8602	1.4713	+15.1
10	15	63–63.5	3.9	0.8602	1.4714	+9.8
11	15	63.5–65.5	3.1	0.8580	1.4725	−10.6
12	15	65.5–67	1.2	0.8576	1.4742	−43.2
13	15	67–70	0.7	0.8533	1.4768	−56.0
14	15	70–72	0.8	0.8508	1.4834	−24.4
15	15	72–91	0.5	0.8535	1.4800	....
16	15	91–97	1.3	0.8862	1.4748	−26.3
17	15	97–107	0.9	0.9062	1.4828	−1.7
18	15	107–110	1.6	0.9494	1.4917	+20.0
19	15	110–115	1.3	0.9518	1.4951	+16.8
20	15	115–118	2.0	0.9497	1.4992	+16.8
21	15	118–119	1.9	0.9429	1.5027	+36.9
22	4	116–125	0.6	0.9486	1.5051	+36.5
23	4	Above 125	0.4			
Pot residue and loss			4.7			

P. PONDEROSA FROM BLACK HILLS, S. DAK.

The crude turpentine had the following characteristics: d_4^{23}, 0.8692; $n_D^{22.5}$, 1.4781; $[\alpha]_D^{23}$, −2.1°.

A batch of 987 Gm. of the turpentine was fractionated in a jacketed and heated Vigreux column 80 cm. long, using a 10:1 reflux ratio.

A portion of fraction 1 was redistilled through a 12-inch glass helix-packed column at atmospheric pressure. The distilling range was 155–168.5° with 12% of the charge remaining undistilled. The refractive index and boiling points of the first two fractions suggested the presence of α-pinene. The second fraction b_{760}, 160–163° yielded a nitrosochloride derivative. The nitrosochloride was twice recrystallized from chloroform by addition of cold methanol, after which it melted at 103°, and showed no depression in melting point on admixture with authentic *dl*-α-pinene nitrosochloride.

β-Pinene was identified in fraction 1 by preparation of nopinic acid m.p. 125.5–126°, which showed no depression in melting point upon admixture with authentic nopinic acid.

Δ³-Carene nitrosate was prepared from fraction 1. After three recrystallizations from chloroform by addition of cold methanol, the nitrosate melted at 147° (with rapid heating) and showed no depression in melting point on admixture with authentic Δ³-carene nitrosate. Δ³-carene nitrosate was also prepared from fraction 3.

The physical properties of fraction 5 indicated that it probably contained *l*-limonene. Fraction 5 was distilled over sodium in a Claisen flask, yielding

a fraction having the following characteristics: b_{760}, 172–175°; n_D^{25}, 1.4770; d_4^{24}, 0.8504. Attempted preparation of a tetrabromide yielded no crystalline derivative.

Fraction 7 was redistilled over sodium in a Claisen flask at 760 mm. yielding two fractions having the following properties: A-b_{760}, 179–181°; d_4^{25}, 0.8518; n_D^{24}, 1.4831; cut B-b_{760}, 181–182°; d_4^{25}, 0.8573; n_D^{24}, 1.4840. A tetrabromide was prenared from cut B. After several recrystallizations from chloroform by addition of cold methanol, the melting point remained at 109–110°. A blue nitrosochloride which melted at 119–120° was also prepared from Cut B. From the properties it is concluded that fraction 7 is a mixture of monocyclic terpenes containing some terpinolene.

Fractions 9 and 10 had a weak odor similar to anise. The densities of these fractions were above the sesquiterpene range indicating the possible presence of methyl chavicol (1, 2). Five Gm. of oil from fractions 9 and 10, 100 Gm. of ice and 3.5 ml. of glacial acetic acid were added to a solution of 3.3 Gm. of potassium permanganate in 200 Gm. of water and the mixture was shaken for three hours. The mixture was made alkaline with potassium carbonate and the manganese dioxide removed by filtration. The filtrate was reduced in volume to 100 ml. and extracted with ether. The ether was evaporated, yielding a brownish crystalline mass. The crude crystals were recrystallized from hot water three times, yielding crystals melting at 184.5–185.5°, which showed no depression upon admixture with authentic anisic acid. The mother liquor was reduced in volume yielding crystals of homoanisic acid which after two recrystallizations from water melted at 82–83°.

Fraction 11 was redistilled over sodium through a 3-inch Vigreux column yielding a heart cut having the following properties: b_{10}, 110–115°; d_4^{24}, 0.928; n_D^{25}, 1.5000; $[\alpha]_D^{25}$, +44.8, M_D, 64.7. A hydrochloride was prepared from 3 Gm. of the heart cut by saturation of an ether solution with dry HCl gas in an ice bath. The hydrochloride (1.5 Gm.) melted at 59–60° after two recrystallizations from glacial acetic acid. Upon admixture with a hydrochloride derived from *d*-longifolene derived from *P. montezumae* from Chiapas, no depression in melting point occurred. Fraction 12 was redistilled over sodium through a 3-inch Vigreux column, yielding a distillate with the following properties: b_5, 119–121°, d_4^{25}, 0.9276; n_D^{25}, 1.5021; $[\alpha]_D^{25}$, +43.8; M_D, 64.8. A hydrochloride (1.2 Gm.) was prepared from 3 Gm. of the distillate. The hydrochloride had the same properties as the hydrochloride derived from fraction 11. Thus, fractions 11, 12, and 13 are mainly *d*-longifolene.

Judging from the molecular refraction (M_D 66.2) fraction 15 contained a bicyclic sesquiterpene. A hydrochloride was prepared from a solution of 1.5 Gm. of fraction 15 in 8 ml. of dry ether by treatment with dry HCl gas for thirty minutes at 0°. The solution stood for a week at 0° and crystals had precipitated from the ether solution. The ether was evaporated and the resulting crystals were recrystallized twice from glacial acetic acid. White plates m. p. 99–109° were obtained. The analysis indicated a monohydrochloride.

Anal.—Calcd. for $C_{15}H_{25}Cl$: C, 74.81; H, 10.47. Found: C, 74.45; H, 10.81.

To sum up, the turpentine of *P. ponderosa* from Black Hills contains 2% *dl*-α-pinene; 38% *l*-β-pinene; 37% Δ³-carene; 3% terpinolene; 1–2% methyl chavicol and 13% *d*-longifolene, and about 1% unidentified bicyclic sesquiterpenes.

P. PONDEROSA FROM PRIEST RIVER, IDAHO

The crude turpentine had the following characteristics: d_4^{25}, 0.8601; n_D^{25}, 1.4763; $[\alpha]_D^{25}$, −2.7. A 733-Gm. sample of the turpentine was fractionated in a 90-cm., 25-mm. inside diameter Todd column packed with glass helices. A reflux ratio of 10:1 was maintained.

Fraction 1 was redistilled through a Todd 90-cm. wire spiral column (5 mm. inside diameter) with a 40:1 reflux ratio. The first 3.8 Gm. received had the following properties: b_{760}, 155–155.5°; d_4^{24}, 0.8486; n_D^{25}, 1.4640; $[\alpha]_D^{24}$, −29.1. *dl*-α-Pinene nitrosochloride was prepared from the low boiling portion of the distillate. After two recrystallizations from chloroform by addition of cold methanol the crystals melted at 106°. A mixed m. p. determination with *dl*-α-pinene nitrosochloride derived from *P. durangensis* showed no depression. The high boiling portion of fraction 1 had the properties of *l*-β-pinene.

Fractions 2 and 3 contain principally *l*-β-pinene. *l*-β-Pinene was identified in fraction 2 by oxidation with alkaline permanganate to sodium nopinate. Ten grams of oil yielded 1.2 Gm. of crude sodium nopinate which was converted to nopinic acid.

After four recrystallizations from benzene the nopinic acid had a melting point of 125° and showed no depression in melting point on admixture with nopinic acid from *P. ponderosa* from McNary, Ariz.

Judging from the physical properties, fraction 4 contained a mixture of Δ³-carene and β-pinene.

The low densities of fractions 5 and 6, along with other physical properties, indicated the possible presence of β-myrcene mixed in with Δ³-carene. Ten grams of oil from fraction 5 was warmed with 2.5 Gm. maleic anhydride for ten minutes. The reaction mixture upon cooling yielded 0.5 Gm. of maleic anhydride crystals which were removed by suction filtration. The mother liquor was mixed with petroleum ether and placed in the refrigerator. The adduct crystallized out but the crystals melted at room temperature. After 7 recrystallizations from petroleum ether b. p. 35–55° in the refrigerator at 0°, 0.4 Gm. of white crystals was received which melted at 34° and further recrystallization did not raise the melting point. The literature value for the maleic anhydride adduct of β-myrcene is 33–34° (3). The U. V. absorption of fraction 5 in 95% ethanol was λ-max. 224.5 which is in agreement with the literature value (9).

Fractions 5, 6, and 7 were predominately *d*-Δ³-carene. *d*-Δ³-Carene was characterized in fraction 7 by preparation of the nitrosate, which after two crystallizations from chloroform by addition of cold methanol had a m. p. of 143° and showed no depression in m. p. upon admixture with authentic Δ³-carene nitrosate.

Fraction 8 was an intermediate one containing both limonene and Δ³-carene. Fraction 9 was redistilled over sodium in a Claisen flask yielding a heart cut possessing the following properties: b_{760}

175–176°; n_D^{21}, 1.4754. A tetrabromide was prepared which, after four recrystallizations from chloroform by addition of cold methanol, had a melting point of 103–104° and showed no depression in melting point on admixture with authentic limonene tetrabromide. Judging from the physical constants, fraction 10 is primarily limonene.

Fraction 12 was evidently a mixture of monocyclic terpene hydrocarbons. From a plot of the physical properties of fractions with densities as ordinates and refractive indices as abscisscae (8, p. 3), the presence of terpinolene was indicated; however, a suitable derivative was not obtained.

Fraction 15 had a mild anise-like odor and a high density suggestive of methyl chavicol. Four grams of fraction 15 was oxidized with potassium permanganate in a manner similar to the treatment of fractions 9 and 10 of *P. ponderosa* from the Black Hills (see above). The acid obtained melted at 81–83° and showed no depression on admixture with authentic homoanisic acid derived from *P. lumholtzii*.

Of fractions 16, 17, and 18 only fraction 18 yielded a solid hydrochloride. Two milliliters of fraction 18 yielded 0.47 Gm. of hydrochloride which melted at 117–118.5° after several crystallizations from glacial acetic acid. The optical rotation of the hydrochloride, dissolved in 95% ethanol containing 11% of chloroform, was: $[\alpha]_D^{24}$, −45°. A mixed melting point determination with a hydrochloride from *d*-cadinene showed no depression.

To sum up the gum turpentine from *P. ponderosa* from Priest River contains about 1–2% *dl*-α-pinene; 12% *l*-β-pinene; 5% β-myrcene; 64% *d*-Δ³-carene; 5% *l*-limonene; 2% methyl chavicol; 4–5% sesquiterpenes, part of which is a cadinene-like sesquiterpene.

P. PONDEROSA FROM MANITOU, COLO.

The crude gum turpentine had the following characteristics: d_4^{23}, 0.8648; n_D^{23}, 1.4761; $[\alpha]_D^{24}$, +1.2°.

A batch of 936 Gm. of the turpentine was fractionated in a jacketed and heated Vigreux column 80 cm. long using a 10:1 reflux ratio.

The physical characteristics of fraction 1 indicated the presence of *dl*-α-pinene. *dl*-α-Pinene was identified in fraction 1 by preparation of *dl*-α-pinene nitrosochloride which melted at 104° and showed no melting point depression on admixture with authentic *dl*-α-pinene nitrosochloride.

β-Pinene was identified in fraction 3 by preparation of nopinic acid which melted at 125° after two recrystallizations from water. No depression of melting point was observed upon admixture with an authentic sample of nopinic acid.

Fractions 4 and 5 had the sweet odor of Δ³-carene. *d*-Δ³-Carene nitrosate, m. p. 144°, and showing no depression of melting point with *d*-Δ³-carene nitrosate derived from *P. ponderosa* from Black Hills, was prepared from fraction 4. *d*-Δ³-Carene nitrosate m. p. 145° was prepared from fraction 6.

The negative rotation and slight lowering in density in fractions 6 and 7 would indicate the probable presence of *l*-limonene, however the crystalline tetrabromide was not isolated.

Judging by the physical constants, fraction 8 contained terpinolene. Fraction 8 was redistilled over sodium yielding two fractions. Cut A, 175–183°; d_4^{24}, 0.8527; n_D^{23}, 1.4848; Cut B, 183–185°; d_4^{24}, 0.8523; n_D^{23}, 1.4840. Pure terpinolene boils at 183–185° and has the constants (8, p. 1) d_4^{25}, 0.8560, n_D^{25}, 1.4864. A tetrabromide was prepared from Cut B and after recrystallization from chloroform by addition of cold methanol melted at 114–115°. Further recrystallizations did not raise the melting point. The melting point was not depressed by admixture with a tetrabromide m. p. 117° prepared from an authentic sample of terpinolene.

Fraction 11 had a weak anise-like odor and a high density which suggested the presence of methyl chavicol. Three grams of fraction 11 was oxidized with potassium permanganate in the manner described for fractions 9 and 10 of *P. ponderosa* from Black Hills (see above). An acid was received which, after several recrystallizations from water, melted at 82–83.5° and showed no depression upon admixture with homoanisic acid.

A portion of Cut B was distilled over sodium, boiling at 125–129° at 17 mm. and having the characteristics: $n_D^{22.5}$, 1.5023; d_4^{23}, 0.932; $[\alpha]_D^{23}$, +45.4. By treatment of 3 ml. of the oil with dry HCl gas in ether solution at 0°, 2.6 Gm. of a hydrochloride was prepared. After two recrystallizations from glacial acetic acid the hydrochloride melted at 59–60° and showed no depression of melting point upon admixture with longifolene hydrochloride derived from *d*-longifolene of *P. oocarpa* from Chiapas.

A hydrochloride was prepared from fraction 15 by treatment of a cold ether solution with dry HCl gas. After two recrystallizations from glacial acetic acid the crystals melted at 118–118.5° and showed no depression in melting point on admixture with cadinene hydrochloride derived from *P. durangensis*.

To sum up, the turpentine of *P. ponderosa* from Manitou contains 2–3% *dl*-α-pinene, 30% *l*-β-pinene; 47% *d*-Δ³-carene; 2–3% terpinolene; 1% methyl chavicol; 8% *d*-longifolene and about 1% *d*-cadinene.

P. PONDEROSA FROM MCNARY, ARIZ.

The turpentine possessed the following physical characteristics: $d_4^{22.5}$, 0.8701; n_D^{23}, 1.4730; $[\alpha]_D^{22.5}$, +13.7°. A batch of 582 Gm. of turpentine was fractionated by means of a 90 cm. long, 25-mm. inside diameter column packed with glass helices. A reflux ratio of 10:1 was maintained. Results of the distillation are shown in Table 1.

Fraction 1 had the properties of *d*- and *dl*-α-pinene. Its presence was proved by preparing *dl*-α-pinene nitrosochloride. After two recrystallizations from chloroform by addition of cold methanol the melting point was 103°. The nitropiperidide was prepared and after three recrystallizations from 90% ethanol melted at 118°. Fractions 2 and 3 are mainly *d*- and *dl*-α-pinene. Fraction 4 is a transition fraction which, when distilled over sodium through a 90-cm. long, 5-mm. inside diameter Todd column at 760 mm. pressure, gave fractions boiling from 156–165°, and their physical properties indicated a mixture of α- and β-pinene. Fraction 5 is of a similar character but has a higher proportion of β-pinene.

Fraction 6 was fractionated over sodium in the 90-cm. long, 5-mm. inside diameter Todd column. Judging from the physical constants the heads

contained some α-pinene and the tails contained Δ^3-carene. A heart cut (22%) had the following properties: b_{760}, 165–166°; d_4^{24}, 0.8609; $n_D^{20.5}$, 1.4772; $[\alpha]_D^{24}$, −16.2°. *l*-β-Pinene was identified in this fraction by preparation of nopinic acid melting at 126°. No depression in melting point occurred upon admixture with authentic nopinic acid.

Fraction 7 was a mixture of β-pinene and Δ^3-carene. Fraction 8 was mainly Δ^3-carene. The nitrosate of Fraction 8 was prepared and after 3 recrystallizations melted at 144.5°. A mixed melting point determination with authentic *d*-Δ^3-carene nitrosate showed no depression. The optical rotation of Fractions 11 and 12 were negative, apparently due to the presence of *l*-limonene, but Δ^3-carene nitrosate was prepared from fraction 12.

Fraction 13 was redistilled over sodium in a Claisen flask yielding a heart cut with the properties b_{760}, 174–177°; $[\alpha]_D^{25}$, −58.2°; $d_4^{25.5}$, 0.8465; n_D^{25}, 1.4785. Preparation of a tetrabromide was attempted but no crystalline derivative was obtained.

Fractions 18 and 19 contained an unidentified sesquiterpene. On attempting to prepare a hydrochloride from fraction 19, no solid derivative was obtained.

From 2 ml. of fraction 21, one Gm. of a crude hydrochloride was obtained by treatment with dry HCl gas in ether solution. After two recrystallizations from glacial acetic acid the crystals melted at 59° and showed no depression on admixture with longifolene hydrochloride derived from *P. ponderosa* of the Black Hills. It is estimated that about 4% of the original turpentine is *d*-longifolene.

To sum up, the gum turpentine from *P. ponderosa* from McNary, Ariz., contains approximately: 34% *d*- and *dl*-α-pinene; 14% *l*-β-pinene; 31% *d*-Δ^3-carene; 4% *l*-limonene (suspected but not identified); 4% *d*-longifolene and 4–5% unidentified sesquiterpene.

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Composition of Gum Turpentines of Pines*

XX. A Report on *Pinus chihuahuana* from Durango, *P. apacheca* from Arizona, and *P. monticola* from Northern Idaho

By N. T. MIROV and P. M. ILOFF, Jr.

Pinus chihuahuana Engelmann is essentially a Mexican pine. It is known under the name of *Pino prieto*, *P. saguaco*, or *P. chamonque* and it occurs as far south as the states of Nayarit, Zacatecas, and the northern part of Jalisco. It grows chiefly in the states of Durango, Chihuahua, and Sonora. In the United States it occurs in southeastern Arizona and southwestern New Mexico. Botanically it is related to *Pinus leiophylla* and *P. lumholtzii* (8). From its discovery in 1848, for over 60 years, Chihuahua pine was considered as a valid species (9); in 1909 Shaw (8) designated it as a variety of *P. leiophylla*. Martinez (5) is of the opinion that *P. chihuahuana* deserves a specific rank. Analysis of turpentine of *P. chihuahuana* reported in this paper seems to substantiate Sudworth's and Martinez's contention: while the turpentine obtained by Mirov (6) from *P. leiophylla* (in Michoacan) consisted of *d*-α-pinene, 90 per cent; Δ^3-carene, 8 per cent; and less than one per cent (if any) of sesquiterpenes. *P. chihuahuana* turpentine was found to consist of *l*-α-pinene, 50 per cent; *l*-β-pinene, 20 per cent; *l*-limonene, 20 per cent; and *d*-longifolene, 8 per cent.

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EXPERIMENTAL

A small sample of *P. chihuahuana* oleoresin, amounting to 420 Gm. was received from C. E. Blanco, El Salto, Durango, Mexico. The amount of turpentine in the oleoresin was 20%.

Physical characteristics of the turpentine were as follows: density, d_4^{24}, 0.8646; index of refraction

n_D^{24}, 1.4730; specific rotation $[\alpha]_D^{23}$, −25.5. A batch of 82 Gm. was fractionated in a 90-cm. Todd Column, having an inside diameter of 12 mm., packed with glass helices. The results of the fractionation are shown in Table I.

The following compounds were identified in the *P. chihuahuana* turpentine: α-pinene in fraction 1. The nitrosochloride, after several recrystallizations from chloroform by means of methanol, possessed a melting point of 105°; β-pinene in fraction 6. Sodium nopinate crystals were obtained in abundance. Nopinic acid was prepared and had a melting point of 125–126°.

Fraction 10 was tested for Δ^3-carene. The results were negative.

Limonene was identified in fraction 15 by preparing limonene tetrabromide which, after three recrystallizations from ethyl acetate, had a melting point of 104–105°.

Longifolene (*dextro-*) was identified in fraction 20 by treatment of 1 Gm. of the oil in absolute ether with dry HCl gas for thirty minutes. Crystals were formed after the treated oil had been kept in a refrigerator for one week and ether was allowed to evaporate on a watch glass. The melting point of the hydrochloride, after it was recrystallized three times from glacial acetic acid, was 59°. It appears from the distillation data that the turpentine of *Pinus chihuahuana* consists of *l*-α-pinene, 50%, β-pinene, 20%, *l*-limonene, 15%, and *d*-longifolene, 5%.

Pinus apacheca Lemm. commonly called Apache pine is also known as *P. mayriana* Sudw., *P. latifolia* Sarg., *P. macrophylla* Engelm., and *P. engelmannii* Carr. All these names indicate that Apache pine is believed by some botanists to be a valid species. Others, like Shaw (8) consider that all these names are mere synonyms of *P. ponderosa*(4). Apache pine grows in southern Arizona and in southwestern and north-central New Mexico. It extends into northern Mexico where it is preferred to be called *P. engelmannii*, Carr. This name also has been recently adopted by the U. S. Forest Service.

The oleoresin used in this investigation was collected in the southern fork of Cave Creek Canyon, Chiricahua Mts., Coronado National Forest in southeastern Arizona, at an elevation of 5,200 feet.[1] The turpentine was obtained by heating a batch of oleoresin *in vacuo*, so that at the end of the distillation, the pressure was 0.1 mm. and the pot temperature reached 210° F.

The gum turpentine of *P. apacheca* had the following characteristics: d_4^{24}, 0.8648; n_D^{24}, 1.4695, $[\alpha]_D^{25}$, +42.0. A 1,000-Gm. sample was fractionated with a 90-cm., 25-mm. inside diameter Todd[2] column packed with glass helices. A reflux ratio of 12:1 was maintained. The results of the fractionation are shown in Table I.

Identification of α-pinene. The physical properties of fraction 2 are close to those of α-pinene. *dl*-α-Pinene nitrosochloride was prepared from fraction 2. After two recrystallizations from chloroform by addition of cold methanol the melting point was 104–105°; mixed with authentic *dl*-α-pinene nitrosochloride it showed no depression of melting point.

Identification of β-pinene. A 7.4-Gm. sample of fraction 5 was oxidized with a basic solution of potassium permanganate and 0.5 Gm. of sodium nopinate was received. The salt was converted into nopinic acid which melted at 125° after two recrystallizations from water; mixed with authentic nopinic acid it showed no depression of melting point.

An attempt to prepare a nitrosate from fraction 6 yielded no crystalline derivative.

A 4.2-Gm. sample of fraction 6 was redistilled over sodium yielding a heart cut (2.3 Gm.) with the following properties: b_{760}, 175–177°; d_4^{23}, 0.8403; n_D^{23}, 1.4735; $[\alpha]_D^{23}$, +12.4. The properties are close to those of dipentene containing a small amount of *d*-limonene. From 1 Gm. of the heart cut 1.5 Gm. of crude tetrabromide was prepared. After two recrystallizations from ethyl acetate white crystals were obtained which melted at 123–124°.

Fraction 11 was redistilled through the 5-mm. inside diameter, 90-cm. long wire spiral column at reduced pressure. The distillation range was 82–101° at 12 mm. About 3 Gm. of material with the following properties was obtained: b_{12}, 91–95°; d_4^{24}, 0.957; $[\alpha]_D^{24}$, +13.6. Although from the properties and odor methyl chavicol was suspected, no crystalline acid was obtained by a permanganate oxidation. For methods consult previous papers of this series.

From 1.7 Gm. of fraction 13, 1.4 Gm. of crude sesquiterpene hydrochloride was prepared. After four recrystallizations from glacial acetic acid the product melted constantly at 59° and showed no depression in melting point on admixture with an authentic sample of longifolene hydrochloride.

Fraction 16 also was shown to be mainly longifolene.

After fraction 16 was received, about 200 mg. of a solid crystallized in the condenser. After several resublimations the white crystals melted at 103–105°. An analysis showed the crystals were 81.49% carbon and 11.48% hydrogen. Calculated for a sesquiterpene alcohol $C_{15}H_{26}O$, the composition would be: C, 80.98%; H, 11.78%.

To sum up, the gum turpentine of *P. apacheca* contained 79–80% *d*- and *dl*-α-pinene, 2% *l*-β-pinene, 2% dipentene, 12% *d*-longifolene plus a trace of an unidentified solid compound.

Pinus monticola D. Don is known as Western White pine. It grows over a wide range extending from Vancouver Island and southern British Columbia through northern Idaho (where it is at its best) to northern Montana and southward through Washington, Oregon, to the Sierra Nevada mountains of California. *Pinus monticola* turpentine, obtained from an isolated locality in northeastern California, had been investigated by Foote and Mirov in 1933 (3). The turpentine was dextrorotatory (α_D, +20.59°) contained *d*-α-pinene, 60%; β-pinene, 26%; *n*-undecane, 1–2%, and unidentified sesquiterpenes. A presence of limonene was suspected. As the separation of the turpentine from rosin was done by steam distillation, it might have been possible that the low boiling heads were not detected. Oleoresin used in the present investigation was obtained from the Priest River Experimental Forest of northern Idaho,[3] i.e., from the

[1] Thanks are due to the administration of the Coronado National Forest for collecting the oleoresin sample for this investigation.

[2] Manufactured by Todd Scientific Co., Springfield, Pa.

[3] Thanks are due to the officers of the Northern Rocky Mountain Forest and Range Experiment Station for procuring the oleoresin at the Priest River Experiment Station in 1948.

TABLE I.—FRACTIONAL DISTILLATION OF TURPENTINES OF P. CHIHUAHUANA FROM DURANGO, P. APACHECA FROM ARIZONA AND P. MONTICOLA FROM NORTHERN IDAHO

Fractions	Pressure, mm.	Boiling Range, °C.	Distillate, %	Density	Index of Refraction	Specific Rotation
		Pinus chihuahuana from Durango (82 Gm. used)				
				$d_4^{24.6}$	n_D^{25}	$[\alpha]_D^{25}$
1	12	44–45	44.6	0.8552	1.4700	−23.4
2	12	45–46	1.0	0.8560	1.4713	−23.0
3	12	46–47	12.0	0.8572	1.4729	−23.0
4	12	47–48	6.0	0.8581	1.4750	−24.5
5	12	48–50	0.6	0.8545	1.4751	−23.0
6	12	50–52	3.4	0.8534	1.4751	−32.0
7	12	52–53	1.6	0.8529	1.4740	−42.3
8	12	53–55	1.6	0.8440	1.4743	−35.0
9	12	55–58	0.4	0.8390	1.4739	−42.7
10	12	58–59	3.4	0.8379	1.4738	−59.7
11	12	59–60	5.8	0.8381	1.4728	−66.8
12	12	60–62	2.4	0.8395	1.4730	−66.8
13	12	62–66	1.6	0.8440	1.4750	−56.8
14	12	66–73	1.6	0.8750	1.4805	−24.1
15	2	70–71	2.4	0.9100	1.4918	−2.2
16	2	71–72	1.6	0.9327	1.4965	+44.0
Residue and Losses			10.0			
		Pinus apacheca from Arizona (1,000 Gm. used)				
				d_4^{23}	n_D^{20}	$[\alpha]_D^{23}$
1	759	121.6–154.6	1.0	0.8627	1.4615	+42.5
2	759	154.6–154.7	53.4	0.8548	1.4660	+45.8
3	759	154.7–155.5	23.6	0.8546	1.4664	+44.2
4	14	45–49	1.9	0.8600	1.4712	+22.0
5	14	49–53	0.8	0.8623	1.4756	0.0
6	14	53–58	0.6	0.8540	1.4742	−1.3
7	14	58–62	0.8	0.8435	1.4749	+8.3
8	14	62–64	0.5	0.8441	1.4760	+11.9
9	14	64–70	0.5	0.8488	1.4797	+8.4
10	14	70–90	1.0	0.8610	1.4877	+1.2
11	14	90–108	1.6	0.9393	1.4933	+13.9
12	14	108–120	2.3	0.9381	1.4985	+44.1
13	14	120–121	2.0	0.9329	1.5033	+52.5
14	14	121	4.6	0.9356	1.5047	+52.3
15	14	121	1.5	0.9339	1.5049	+49.3
16		Above 121	0.5	0.9277		+53.4
Residue and Losses			3.4			
		Pinus monticola from northern Idaho (346 Gm. used)				
				d_4^{23}	n_D^{23}	$[\alpha]_D^{23}$
1	760	130–155	2.1	0.8145	1.4511	..
2	760	155–158	14.4	0.8513	1.4634	−23.1
3	760	158–159	1.1	0.8536	1.4660	..
4	760	159–160	21.2	0.8554	1.4680	−28.0
5	760	160–163	12.0	0.8577	1.4694	−28.9
6	760	163–164	4.3	0.8562	1.4701	−29.5
7	760	164–167	6.2	0.8560	1.4714	−33.2
8	760	167–171	12.1	0.8548	1.4722	−38.8
9	16	51–56	3.8	0.8608	1.4744	−26.3
10	16	56–64	1.9	0.8528	1.4732	−33.3
11	15	64–68	0.8	0.8465	1.4735	−61.1
12	15	65–66	2.8	0.8410	1.4727	−91.6
13	15	66–71	0.8	0.849	1.4754	−75.4
14	15	71–76	1.2	0.8425	1.4762	−24.0
15	15	76–84	1.4	0.8066	1.4517	−1.0
16	15	84–95	0.3	0.8157	1.4469	..
17	15	95–105	1.9	0.9112	1.4659	−13.4
18	15	105–114	0.5	0.9180	1.4723	..
19	15	114–122	1.1	0.9071	1.4808	..
20	15	122–134	1.0	0.9022	1.4902	..
21	15	134–138	0.7	0.8954	1.4948	..
22	4	140–150	1.1	0.8954	1.5031	..
Residue and Losses			7.3			

area of the optimal development of this species. The turpentine was obtained by heating oleoresin under reduced pressure so that at the end of distillation, the temperature reached 180° and the pressure was 0.1 mm. of mercury. Yield of turpentine was 20.0%.

The physical characteristics of the turpentine of *P. monticola* were as follows: d_4^{23}, 0.8500; n_D^{23} 1.4687; $[\alpha]_D^{23}$, −28.8.

A batch of 346 Gm. of the turpentine was fractionated with a 90-cm. long, 12-mm. inside diameter Todd column packed with 3/32-inch single-turn glass helices. A reflux ratio of 10:1 was maintained. The results of the distillation are shown in Table I.

The physical properties of the first fraction suggested the presence of *n*-heptane. The oil from the dry ice trap was shaken repeatedly with fuming sulfuric acid until the acid was no longer darkened by the oil. After washing and drying the oil weighed 2 Gm. The properties of the clear light liquid are compared with *n*-heptane below:

	n-Heptane from trap	*n*-Heptane[a]
n_D	1.3881^{23}	1.3876^{20}
d	1.06807_4^{23}	0.6873_4^{20}
B. P.	98.5[752]	98.4[760]

[a] Rossini, *et al.*, "Selected values of properties of hydrocarbons," Circular of the National Bureau of Standards C461, U. S. Govt. Printing Office, 1947, p. 39.

dl-α-Pinene nitrosochloride was prepared from fraction 2. The nitrosochloride was recrystallized twice from chloroform by addition of cold methanol after which it melted at 103–104°. It showed no depression in melting point on admixture with authentic *dl*-α-pinene nitrosochloride. *dl*-α-Pinene nitrolpiperidide was prepared from the nitrosochloride. After two recrystallizations from 90% alcohol, the compound melted at 118.5–119.5° and showed no depression with authentic *dl*-α-pinene nitrolpiperidide.

A 20-Gm. portion of fraction 5 was oxidized with alkaline permanganate to sodium nopinate. The sodium nopinate was converted to nopinic acid which melted at 126° after recrystallization from benzene alternated with water; mixed with authentic nopinic acid, it showed no depression in melting point.

A heart cut from fraction 12 redistilled over sodium had the following properties: b_{760}, 173.5–174.5°; n_D^{25}, 1.4727; d_4^{23}, 0.8410. A tetrabromide was prepared from the heart cut. After several recrystallizations from methanol the tetrabromide melted at 103° and showed no depression in melting point on admixture with authentic *l*-limonene tetrabromide.

The unsaturated compounds were removed from fraction 15 by means of fuming sulfuric acid. The properties of the oil received compared with *n*-undecane are as follows:

	n-Undecane from *P. monticola*	*n*-Undecane (2)
n_D	$1.4181^{20.3}$	1.41902^{20}
d	0.7394_4^{23}	0.7404^{20}
B. P.	197[752]	195.8[760]

Fraction 17 had a sweet pine-like odor. A sample was analyzed giving the following results: C, 74.45%, H, 10.81%. This result suggests a terpene ester. A sample of 2 ml. of the ester was saponified. The acid fraction had the following Duclaux constants: 1–7.4, 2–7.4, 3–8.1. These are close to the values for acetic acid. A *p*-bromophenacyl ester of the acid was prepared and recrystallized several times until it melted at 82–83° (lit. 85 for the acetic acid derivative).

A 3,5-dinitrobenzoate melting at 154–154.5° was prepared from the alcohol fraction of the saponified ester. From the 3,5-dinitrobenzoate, an α-naphthylamine addition product melting at 139° was made. The literature value for the 3,5-dinitrobenzoate of *d*-borneol is 154° (7), and for the α-naphthylamine addition product is 140.5° (1).

Judging from the physical constants the sesquiterpene portion is predominantely a mixture of a bicyclic and a monocyclic sesquiterpene. Fraction 20 was redistilled over sodium metal at reduced pressure. The distillate had the following physical properties: d_4^{24}, 0.8971; n_D^{25}, 1.4918; $[\alpha]_D^{25}$, +5.3.

On preparation of a hydrochloride two hydrochlorides were isolated. The first to crystallize from glacial acetic acid was in the form of needles melting after several recrystallizations from glacial acetic acid at 117°. The second to crystallize was in the form of plates melting after two recrystallizations at 75–79°. It is possible that cadinene and bisabolene are present in the oil since they frequently occur together and have hydrochlorides which melt at 118.5° and 79°, respectively. Lack of material prevented further investigation.

To sum up, *P. monticola* from northern Idaho contained 2–3% *n*-heptane, 32% *l*-and-*dl*-α-pinene, 45% *l*-β-pinene, 7% *l*-limonene, 2% undecane, 2% bornyl acetate, 4–5% sesquiterpenes of which one is monocyclic and another bicyclic and 7% pot residue and loss.

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Note

A Note on Cleaning Culture Tubes for the U. S. P. Vitamin B_{12} Microbiological Assay*

By E. M. STAPERT and L. STUBBERFIELD

THE MICROBIOLOGICAL ASSAY for vitamin B_{12} U. S. P. XIV (1) has proved to be consistent and very satisfactory in our laboratories for the past year. Previously we encountered erratic results which we believed were attributable to the method used in cleaning the culture tubes.

Water, various detergents, and sulfuric acid (65% v/v) have been used in our laboratories for cleaning culture tubes. Cleaning methods were as follows: *Water.*—Immediately after use the tubes were rinsed in an inverted position with hot, cold, and deionized water for ten minutes each rinsing, and dried by hot air. *Detergent.*—Tubes were allowed to stand two hours in a detergent solution prepared according to manufacturer's directions. The tubes were then drained, rinsed, and dried as above. *Sulfuric acid (65% v/v).*—Tubes were allowed to stand two hours in acid solution, then drained, rinsed, and dried.

Inconsistent results when encountered usually followed tube cleaning by water or detergents and seldom followed acid cleaning. To verify these observations new tubes were selected and grouped into three sets, reserving one set for each of the three cleaning methods. U. S. P. vitamin B_{12} standards were run repeatedly in each set until erratic results occurred. The U. S. P. procedure was modified by using duplicate dose levels and determining growth turbidimetrically after twenty hours' incubation.

Figure 1 shows that erratic results were obtained after the second detergent and third water cleaning. The acid-cleaned tubes were satisfactory after the third cleaning. Loy, *et al.* (2), have reported that detergents used in their laboratories inhibited growth of *L. Leichmannii* 7830. Since our previous experience with detergents had varied from erratic results after one cleaning to good results following five, we considered the effect of residual detergents on the assay. One drop of a detergent solution was added to each tube of a U. S. P. vitamin B_{12} standard. This standard and a control were identical, proving that detergents used here do not contribute to the erratic response.

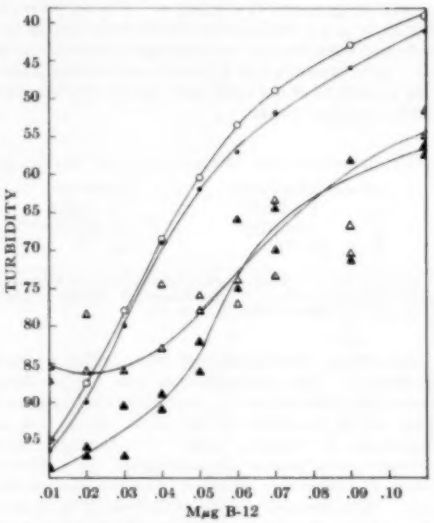

Fig. 1.—U. S. P. vitamin B-12 standard curves from: ●–new tubes; ○–acid cleaned three times; ▲–detergent cleaned two times; Δ–water cleaned three times.

The use of acid-cleaned tubes for the past year has eliminated a last serious inconsistency in this assay. Acid-cleaned tubes are required only for the U. S. P. vitamin B_{12} assay. Detergent-cleaned tubes may be used in other microbiological assays including riboflavin, pyridoxine, pantothenic acid, pantothenol, nicotinic acid, folic acid, biotin, and inositol.

* Received February 23, 1954, from the Biological Control Laboratories of the Upjohn Company, Kalamazoo, Mich.

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Book Notices

Antibiotics Annual, 1953–54. Proceedings of the Symposium on Antibiotics. U. S. Dept of Health, Education, and Welfare. Food and Drug Administration. Medical Encyclopedia, Inc., 1953. 632 pp. 18 x 26.5 cm. Price $8.

During the past several years the Division of Antibiotics of the Food and Drug Administration has sponsored an annual symposium designed to orient the scientific worker in the current status of antibiotic therapy and development. The 1953–54 Symposium was sponsored jointly by the Department of Health, Education, and Welfare, the Division of Antibiotics of the Food and Drug Administration, with the collaboration of the journal *Antibiotics and Chemotherapy* and was held in Washington, D. C., on October 28, 29, and 30, 1953.

During the three-day program more than 100 papers were presented on recent advancement in the development of new antibiotics and new knowledge concerning their applications as therapeutic agents and animal food supplements. All of the papers presented during the Symposium have been collected, published, and made available for practically an up-to-the-minute survey on developments in the antibiotic field during the preceding year. The editors and the publisher are to be congratulated upon the excellent service rendered in making the papers available in a relatively short time after the Symposium. The book is provided with an adequate subject index which greatly facilitates its use when one is interested only in a limited number of the subjects covered by the Symposium papers.

The Dynamics of Virus and Rickettsial Infections. By FRANK W. HARTMAN, FRANK L. HORSFALL, Jr., and JOHN G. KIDD, Editors. The Blakiston Company, Inc., New York, 1954. xii + 461 pp. Ill. 15.5 x 23.5 cm. Price $7.50.

This volume includes papers and discussions which were presented to an International Symposium on the Dynamics of Virus and Rickettsial Infections, held in Detroit, Mich., in October, 1953. The objectives of the Symposium were to provide for an exchange of ideas and information between workers in different areas of the virus and rickettsial fields. The subjects discussed were grouped into five major categories: Mechanisms of Virus and Rickettsial Infections, which included twelve papers; Ecology and Pathogenesis of Virus and Rickettsial Infections, with six papers; Mechanisms of Immunity in Virus and Rickettsial Infections, with six papers; Laboratory Diagnosis of Virus and Rickettsial Infections, with five papers; and Approaches to Prophylaxis and Therapy of Virus and Rickettsial Infections, with six papers. The several papers reproduced in the five main categories present much useful information of a general nature relating to virus and rickettsial organisms. Twenty of the papers are concerned mainly with studies on viruses infecting man or animals, seven with viruses infecting bacteria, three with viruses infecting plants, and three with rickettsiae. All of the papers are documented with references to the original literature, but unfortunately the book is not provided with an index. To the specialist in the fields covered this is probably of minor importance, but to a reader with only a general interest in the subject, an index would be valuable.

Grundlagen der Pharmakologie für Apotheker, Chemiker und Biologen. By K. W. MERZ. 5th rev. ed. Wissenschaftliche Verlagsgesellschaft m.b.H., Stuttgart, 1953. 371 pp. 89 illustrations. Price *DM* 28.50 (about $7).

This is a very well-known text in Germany and is now in its fifth edition. It is written for students of pharmacy, chemistry, and biology. The author is well qualified to write such a book since he holds a Ph.D. degree in pharmaceutical chemistry and an M.D. degree.

It is an established fact that today's pharmacy student has to be thoroughly familiar with drug action. This book presents to the student an introduction to pharmacology in a clear, concise form. The pharmacy curriculum in Germany does not require the student to have a fundamental knowledge of physiology or anatomy. It is, therefore, very important that a textbook will convey a clear understanding of pharmacologic action on the basis of physiological function. This is accomplished by illustrations and excellent drawings which supplement the text. Emphasis is placed on chemical composition. A final chapter on toxicology is of particular importance in this age of industrialization.

This book is a valuable addition not only for the pharmacist, but also for the chemist who, in his search for new synthetic remedies, must be familiar with drug action.

Introduction to Pharmacy. 3rd ed. HENRY M. BURLAGE, Editor. Co-authors: Burt, Lee, and Rising. McGraw-Hill Book Co., New York, 1954. 710 pp. 15 x 21.5 cm. Price $9.50.

By the time a book has passed through two revisions within a 10-year period, most educators are familiar with its scope and purpose. The first edition of *Introduction to Pharmacy* was published in 1944. It was originally designed to introduce the student of pharmacy to the professional subjects he will pursue throughout his academic program and to orient him in regard to the professional and commercial aspects, requirements, and opportunities in pharmacy. The design of the book has not been changed, but the third edition has been thoroughly revised to conform with changes in U. S. P. XIV and N. F. IX, and to present recent physical and chemical aspects of modern pharmaceutical technology by the addition of much new material on solutions and physical chemical methods of operation. The book also includes a number of new illustrations of apparatus and new references for collaborative readings.

Methods in Medical Research. Vol. 6. M. MURRAY STEELE, Editor-in-Chief. The Year Book Publishers, Chicago, 1954. 271 pp. 14.5 x 22 cm. Price $7.

This latest volume of *Methods in Medical Research* is divided into four sections Two of these sections may be of special interest to pharmacologists and biochemists working in pharmaceutical areas of research and application. These two sections, the third and fourth, are devoted to statistics in medical research and include discussions of sampling, planning, confidence limits, standards of significance, standard deviations and standard errors, sample sizes, and the value of consultation with a statistician before applying statistical methods to the results obtained from a research problem. Section IV is devoted to a complete discussion of the design and construction of metabolism cages. In the first two sections, some methods of studying human genetics and procedures used in environmental medical research are covered. The book is provided with a subject index, is well illustrated, and the printing and binding are of the same quality as in previous editions.

Lehrbuch der Organischen Chemie. 12th ed. Revised. By PAUL KARRER. Georg Thieme Verlag, Stuttgart, Germany, 1954. 949 pp. Illustrated. 19 x 27 cm. Price *DM* 59.70 ($14.20). (In the U.S.A. and Canada, Intercontinental Medical Book Corporation, New York.)

Those who have used a textbook through several editions always look forward with interest to new editions, and this edition of *Lehrbuch der Organischen Chemie* will afford particular pleasure to former users of this book. This edition has been considerably revised as can be seen from the reduction in the number of pages from 1080 pages in the 11th edition to 949 pages in the 12th edition. The condensation has been accomplished, however, by a careful revision of the material and not by elimination of any entire chapter.

The aim and purpose of this textbook was to provide students with a textbook of organic chemistry of medium size which would give them a survey of the ever-increasing body of facts. To make the problems of organic chemistry more easily understood and to make the subject more real and live, special attention has been paid in all chapters to the description of methods of synthesis and of determining the constitution of organic compounds. The methods of producing the majority of the compounds dealt with are described and the proofs of their constitution and configuration are thoroughly discussed.

The arrangement of the subject matter has been determined only by didactic considerations. The historical divisions into aliphatic, carbocyclic, and heterocyclic compounds are closely followed, except where it was deemed advisable to discuss together certain classes of compounds which are closely related. In each section the compounds are regarded as functions of the hydrocarbons, and have been arranged, as far as possible, according to their functional groups (monovalent, divalent functions, etc., halogen function, hydroxyl function, etc.).

References are well selected and include those up into 1953. It is hoped that, as in the past, an English translation of this book will soon be available.

A textbook of moderate size must be limited in certain directions in order to discuss more fully other questions. In this book, the chemistry of natural products and biochemical and medicinal chemicals have been emphasized. As the book deals with the more important biological compounds of interest it should be of value to advanced students of pharmacy and medicine.

In this edition a large amount of space has been given to reaction mechanisms and electronic theories. Many of the tables of scientific and statistical materials, formerly appended to the book, have been incorporated into the various chapters.

Organic Chemistry. 2nd ed. By REYNOLD C. FUSON and H. R. SNYDER. John Wiley & Sons, Inc., New York, 1954. 544 pp. 15.5 x 24 cm. Price $6.50.

This book is designed to present an accelerated introduction to organic chemistry and so will be of interest to those who prefer this method. The authors devote approximately the first third of the book to the structure and behavior of the principal organic molecules, thus affording the reader a basic scope of organic chemistry, necessary for the more advanced treatment of topics in the remaining sections. The subject matter is arranged so that the book is suitable for either a two-semester or for a three-quarter course.

Theoretical considerations are treated extensively, and reaction patterns, both general and specific, are emphasized. Cross referencing of the reactions serves to eliminate duplication and also avoids the introduction of some reactions out of context.

As many organic medicinals are found throughout the book and several chapters dealing with biochemistry are included, it should be of value to students in these fields.

Suggested readings given at the chapter endings will be useful to those needing additional material on many of the subjects.

Infrared Absorption Spectra of Steroids, An Atlas. By KONRAD DOBRINER, E. R. KATZENELLENBOGEN, and R. NORMAN JONES. Interscience Publishers, Inc., New York, 1953. xiv pp. and 308 spectra. 16 x 24 cm. Price $11.50.

A brief introductory portion of the book reviews experimental techniques for the determination of infrared spectra. The relation between spectra and molecular structure is discussed. The major portion of the book is devoted to 308 charts showing infrared spectra of steroids such as: C_{18} steroids (e. g., estradiol-17° diacetate), C_{19}-29 steroids (e. g., androstane), bile acid esters, steroid sapogenins and derivatives, and steroid alkaloids. A bibliography, numerical, and alphabetical indexes of the charts are appended. This book is a valuable source of references for chemists, physicists, and clinical analysts.

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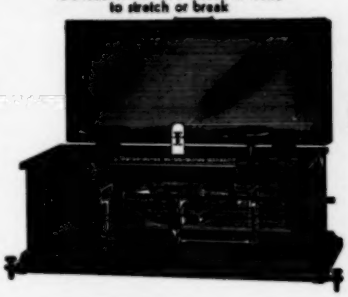

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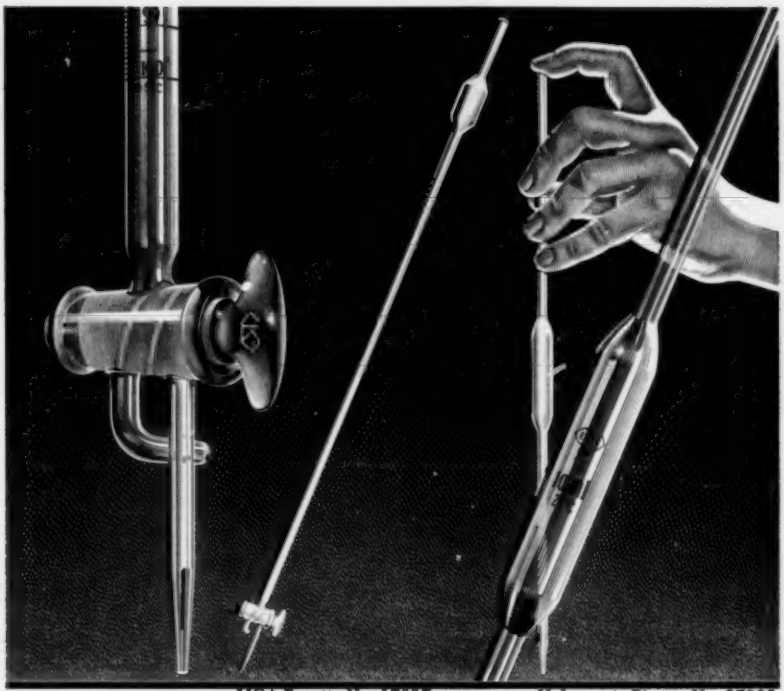

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